Systemic Platelet Activation in Mice Exposed to Fine Particulate Matter

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Contract number: 07-337

February 2011

"Prepared for the California Air Resources Board and the California Environmental Protection Agency"

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Acknowledgement:

The following key personnel were instrumental in conducting the studies described herein: Dr. Dennis Wilson, Dr. Michael Lame, Dr. Hnin-Hnin Aung, Dr. Laura den Hartigh, Dr. Jeffrey Norris, and Monica Pombo.

This Report was submitted in fulfillment of contract number 07-337, “Systemic Platelet Activation in Mice Exposed to Fine Particulate Matter” by Fern Tablin, School of Veterinary Medicine, University of California, Davis under the sponsorship of the California Air Resources Board. Work was completed as of January 2011.

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Abstract:

We conducted five separate experiments designed to define the effects of ambient particles on the cardiovascular system. Mice were exposed to concentrated ambient particles (CAPs) less than 2.5 microns in diameter (PM2.5) or instilled with PM 2.5 from the Fresno, California area. Control animals were exposed to filtered air or physiological saline. Studies were conducted during summer and winter in both urban and rural areas. The three CAPS experiments were conducted in winter 2008, summer 2008, and winter 2009, and evaluated the effects of CAPs on the vascular and hemostatic systems. Later studies examined the effects of short term *in vivo* instillation of PM 2.5 that was collected during the CAPS experiments. In all studies, we determined the production of systemic inflammatory cytokines to evaluate the extent of pulmonary, coronary and systemic inflammation. Platelet upregulation and activation in response to agonist was evaluated by flow cytometry. We determined the expression and activation of key platelet integrins and the extent of alpha granule and lysosomal granule secretion. Complete blood counts were performed. Lung tissue was evaluated for changes in gene expression either by gene array or by laser capture micro-dissection. Particle exposed animals showed platelet activation in all studies compared to control animals, while proinflammatory cytokines were predominantly upregulated in the winter CAPs and winter instilled exposures. Exposure to winter particulate matter (PM) appears to activate different physiological outcomes than summer exposures.

Executive Summary:

Background:

California's San Joaquin valley air basin is a flat inland valley with severe degradation of air quality by ozone and particulate matter (PM). It is classified as a non-attainment region for both state and national PM2.5 standards (1, 2). Exposure to fine and ultrafine PM (≤2.5 micron or ≤0.1 micron in diameter, respectively) has been associated with exacerbations of respiratory ailments such as asthma and chronic obstructive pulmonary disease (COPD), often correlated with exposures of at least 1-2 days (Braga et al., 2001a). An increasing body of evidence suggests that effects of such exposures are not limited to pulmonary effects, but also include increased incidence of morbidity and mortality associated with cardiovascular disease (CVD) as early as 1-3 days following exposure (1996 Health Effects of Outdoor Air-Pollution, Brook et al., 2004, Schwartz 1999, Peters et al., 1999), or even within a few hours (Peters et al., 2001). The cardiovascular effects of PM inhalation potentially operate through inflammatory or thrombotic mechanisms (Calderon-Garciduenas et al. 2003, Gilmour et al., 2005). We have recently shown that mice respond to inhalation of concentrated ambient PM from Fresno, CA by exhibiting a pro-inflammatory and pro-coagulant state after 2 weeks of exposure (Wilson et al., 2010). However, despite substantial epidemiological and experimental evidence, the mechanisms by which PM exposure induces systemic inflammation and cardiovascular effects remain unknown.

Ambient PM is a mixture of salts, crystal elements and carbonaceous aggregates that may contain metals, adsorbed polycyclic aromatic hydrocarbon (PAH) compounds, and biologically active material such as endotoxin. Fine (PM2.5) and ultrafine (UFP) particles become easily deposited and retained in the alveoli and small airways (diameters of less than 1 mm) (Kreyling et al., 1999), induces pulmonary inflammation (Li et al., 1999), and elicits oxidative stress (Brown et al., 2001, Li et al., 2003). Experimental studies suggest that UFP can cross the lung epithelial barrier to enter the circulation (Nemmar et al., 2001, Nemmar et al., 2002, Geiser et al., 2005). Indeed, it has been demonstrated that even a brief exposure (2 hours) to fine particulate air pollution can result in increased frequency of myocardial infarction (Peters et al. 2001). Data from a large European multi-city study demonstrated an even greater mortality rate associated with particulate matter less than PM10 levels in a distributed lag model of 40 days compared with short-term one and two day analysis (Zanobetti et al. 2002), but whether this occurs to an extent sufficient to elicit systemic inflammation remains uncertain. Thus it is critical to develop a more in-depth understanding of the interactions between fine particular matter and the cardiovascular system. Our hypothesis is that

there are different physiological cardiopulmonary responses to CAPs exposures in the winter than in the summer. Furthermore, we hypothesize that PM from urban and rural sites also have differing physiological effects.

Methods:

Mice were exposed either to CAPs (PM 2.5) or filtered air (control group) for two week periods during the summer and winter of 2008 and winter of 2009. CAPS exposures were performed in the Fresno, CA area. An additional series of mice received intra-tracheal instillation with either saline (control group) or PM 2.5 in saline collected during the summer 2008 urban Fresno or winter 2008 rural Fresno CAPS exposures. We compared responses to exposures by both season (summer vs. winter) as well as by location (urban vs. rural). Concentrated fine and ultrafine ambient particles for animal exposures were obtained using a portable particle concentrator (Versatile Aerosol Concentration Enrichment System or VACES) (Kim et al., 2001a, 2001b). By incorporating size-selective inlets, the VACES provides CAPs in carefully defined size ranges. We used a 2.0μm cut-off cascade impactor based pre-filter to expose mice to a mixture of fine and ultrafine particulates.

Particles for intra-tracheal experiments were collected with High Volume Samplers. The PM collected for biological testing during the CAPS exposures was collected on High Volume (1 m^3/min) samplers co-located with the inlet for the VACES and the other MOUDI samplers and low volume (0.01 m3/min) filter samplers. The High Volume samplers were equipped with an inlet that separately collected PM2.5 vs. PM10-2.5.

Platelet activation was measured by flow cytometry using well established methodologies from the Tablin laboratory. Cytokine measurements – both from serum and lung were conducted using a bio-plex multi-bead ELISA systems to evaluate for both pro-inflammatory and pro-coagulant cytokines as previously described in Wilson et al. (2010). Laser capture studies and gene array studies were conducted using previously established technologies.

Results:

There were locational and seasonal differences in the responses of mice to either CAPs or intra-tracheal instillation of CAPs. The greatest pro-coagulant and pro-inflammatory responses were seen in the winter 2008 rural CAPs studies, with similar although less extensive responses seen in response to winter urban2009 exposure. Interestingly, resting, unstimulated platelets were more activated and showed a greater response to agonist in all winter CAPs exposures than in summer CAPs exposures. Summer CAPs studies demonstrated platelet activation with limited cytokine upregulation, consistent with TH-2 and allergic responses. Intra-tracheal studies with summer 08 particles were similar to those of the summer 09 CAPs studies, while intra-tracheal studies with winter

urban 09 particles were similar to Winter CAPs studies, indicative of a greater inflammatory response. Laser capture studies demonstrated that exposure to Winter PM resulted in increased expression of cytochrome P450, family 1, member A1 (CYP1A1) and keratinocyte chemoattractant (KC), while those in the summer had decreased CYP1A1 expression but some limited indications of endothelial cell activation as indicated by increased E-selectin and intra-cellular adhesion molecule-1 (ICAM-1) expression. These observations were confirmed in our intra-tracheal experiments.

Conclusions:

Exposure to PM generated during winter appears to have different physiological consequences than exposure during summer. Platelet activation was seen in all of the studies, while increased pro-inflammatory cytokines were largely limited to winter exposures. Further studies are required to define which components of the ambient PM mixture are most responsible for these effects.

Introduction:

Particulate matter in the atmosphere is a complex mixture of individual particles derived from a variety of natural and anthropogenic sources. Standard PM size fractions are typically categorized into coarse (2.5-10μm) fine (<2.5μm) and ultrafine (< 0.1μm) ranges. While these size ranges are important in helping determine environmental policy, there is also a growing body of evidence that suggests that particle sizes may have differential health effects. Ultrafine particles have also been shown to have a greater surface area and may have more toxic potential than larger particles (Oberdorster 2001; Donaldson et al. 2001). California's San Joaquin valley air basin is a flat inland valley with severe degradation of air quality by ozone and particulate matter (PM). It is classified as a non-attainment region for both state and national PM2.5 standards (Cox et al., 2009, Allen and Turner 2008).

Epidemiological studies have shown that episodes of elevated particulate matter (<10μm) are associated with increased morbidity and mortality partly due to respiratory causes, but most prominently due to cardiovascular causes (Dockery et al. 1993; Samet et al. 2000;Schwartz 1994). While pulmonary effects tend to have a "lag" period of one or two days post-exposure, cardiac effects are more immediate (Braga et al. 2001a). Indeed, it has been demonstrated that even a brief exposure (2 hours) to fine particulate air pollution can result in increased frequency of myocardial infarction (Peters et al. 2001). An associated issue is whether this increased frequency of myocardial infarction subsequent to PM exposure is simply a result of hastened death of patients who are terminally ill. Data from a large European multi-city study demonstrated an even greater mortality rate associated with PM10 levels in a distributed lag model of 40 days compared with short-term one and two day lags (Zanobetti et al. 2002).

Studies of PM exposure and subsequent exacerbation of disease often lack analysis of cardiac pathology. While some studies used ischemic heart disease or myocardial infarction statistics, others report all causes of cardiovascular deaths generally. In one study where specific cardiovascular causes of death were evaluated, 70% of the increase in cardiac deaths was due to myocardial infarction (Braga et al. 2001a). A more recent study confirmed the strong association between fine PM and risk of myocardial ischemia, and demonstrated that PM was more highly correlated with cardiac rather than pulmonary associated mortality (Pope et al. 2004). Despite epidemiologic associations (Ruckerl et al. 2007), as well as *in vivo* studies, the precise mechanism by which acute PM exposure leads to cardiac mortality remains to be elucidated. Several studies have suggested that the cardiovascular effects of PM inhalation potentially operate through inflammatory or prothrombotic mechanisms (Calderon-Garciduenas et al. 2003, Gilmour et al. 2005). Furthermore, we have recently shown that mice respond to inhalation of concentrated ambient PM from Fresno, CA by

exhibiting a pro-inflammatory and pro-coagulant state after two weeks of exposure (Wilson et al. 2010).

The response to environmental particulate matter, based on the more extensive pulmonary studies, has focused on up-regulation of inflammatory cytokines such as IL-6 or induction of oxidative stress and associated response elements (Becker et al. 2005, Mutlu et al. 2007). Studies of the cytokines expressed in subjects exposed to high levels and a wide variety of PM10 pollutants demonstrated that several pro-inflammatory cytokines are expressed (van Eeden et al. 2001). These subjects showed significant increases in serum of interleukin-6 (IL-6), interleukin-1β (IL-1β), macrophage inflammatory protein-1α (MIP-1 α) and granulocyte-macrophage colony-stimulating factor (GM-CSF) leading the authors to suggest that acute exposure to air pollution induces a systemic inflammatory response contributing to cardiopulmonary disease. It should be noted, however, that a number of other similar studies failed to demonstrated release of systemic cytokines in response to ultrafine PM exposure (Ghio et al. 2000; Elder et al. 2004; Smith et al. 2006).

Platelets express a number of chemokine receptors including, but not limited to C-C chemokine receptor type 1(CCR1), type 3 (CCR3), type 4 (CCR4), C-X-C chemokine receptor type 4 (CXCR4), and C-X-C3 receptor type 1(CX3CR1) (Clemetson et al. 2000). These receptors have a pleotropic group of ligands including some of the inflammatory cytokines noted above. Activation of these receptors by pro-inflammatory cytokines can serve to prime or up-regulate platelets without directly inducing platelet aggregation. Priming results in platelets that are more responsive to physiological agonists such as thrombin, thereby establishing a procoagulant environment on a systemic level.

Our hypothesis for these studies is that exposure to PM2.5, either by inhalation CAPs or by intra-tracheal instillation results in the up-regulation of platelets and systemic cytokines. Platelet activation, and/or interaction of platelets with monocytes would be consistent with a pro-coagulant environment, and increased systemic pro-inflammatory cytokines would further support the hypothesis.

Methods:

Experimental Methods

PM Collection: The San Joaquin Valley Aerosol Health Effects Center (SAHERC) rural sampling location is at the University of California WestSide Research and Extension Center (WSREC) outside Five Points (Fresno County) California, approximately 35 miles southwest of Fresno, and 10 miles east of Interstate 5 on the west side of the San Joaquin Valley. The facility actively operates as an agricultural research station with

mixed crop plantings. The SAHERC urban sampling location is located in downtown Fresno (550 E. Shaw Ave.). The choice of locations was designed to enable comparison between responses to PM2.5 from an urban setting with predominantly un-aged highway emissions and a downwind location characterized by an aged PM2.5 mixture. The exposures described in this study were done in February 2008 (rural winter) and July 2008 (urban summer) as well as January 2009 (urban winter).

Concentrated fine and ultrafine ambient particles for animal exposures were obtained using a portable particle concentrator (Versatile Aerosol Concentration Enrichment System or VACES) (Kim et al., 2001a, 2001b). By incorporating size-selective inlets, the VACES provides CAPs in carefully defined size ranges. We used a 2.0μm cut-off cascade impactor based pre-filter to expose mice to a mixture of fine and ultrafine particulates.

The PM collected for biological testing (such as that used in our intra-tracheal studies) during the CAPS exposures was collected on High Volume (1 m3/min) samplers co-located with the inlet for the VACES and the other MOUDI samplers and low volume (0.01 m3/min) filter samplers. The High Volume samplers were equipped with an inlet that separately collected PM2.5 vs. PM10-2.5. Sections of filters sonnicated in water with a probe sonnicator and samples concentrated to a final concentration of 200 μg/ml approximately the same amount of PM2.5 inhaled during the two week CAPs studies.

Mice: For CAPs studies, male C57Black mice were housed eight to a cage in polycarbonate inhalation chambers. Each chamber had an independent flow regulator, and the flow from the four chambers was equal to the output of the VACES. Mice were exposed six hours (10 AM to 4 PM) per day five days a week for two weeks in a temperature and humidity controlled mobile exposure module. Following the final exposure, mice were transported to our research laboratories and euthanized the following morning, approximately 16 hours after the last exposure. For this particular exposure, complete blood counts (CBC) were obtained from a second group of eight control and eight exposed mice from the same exposure regimen that were euthanized 36 hours after the end of exposure. For intra-tracheal studies we used BALB C male mice, each one housed individually. We chose these animals for the intratrachael studies due to their larger size and more robust nature. For intra-tracheal instillations, mice were sedated with ketamine/xylazine and PM was introduced into the trachea in between tracheal rings through a 32 gauge needle. Animals received approximately 200μg of PM in 30μl of sterile saline. Control animals received 30μl of sterile saline.

Exposure monitoring: Continuous particle number concentrations were measured in the ambient air using a Scanning Mobility Particle Sizer (SMPS) (0.01-0.7μm) and an Aerodynamic Particle Sizer (APS) (0.5-10μm). Continuous particle scattering was measured in the exposure chambers for each animal inhalation experiment using the

DataRAM nephelometer (RAM-1 Monitor, MIE Inc., Billerica, MA) and correlated to filter-based gravimetric measurements to provide a continuous time series of particle mass. Bulk PM1.8 samples were collected with 3 co-located Reference Ambient Air Monitor (RAAS) filter samplers loaded with prebaked (48hrs@550^{o}C) quartz fiber filters and Teflon membrane filters. Size-resolved PM samples were collected with six identical Micro-Orifice Uniform Deposit Impactors (MOUDIs) with size cuts for 0.056, 0.1, 0.18, 0.32, 0.56, 1.0, and 1.8 μm aerodynamic diameter particles. All the MOUDI stages can be summed to calculate PM1.8 mass for comparison to co-located RAAS samples; the final impaction stage of each MOUDI collects particles entirely in the ultrafine size range (PM0.1). A cyclone separator was used upstream of each MOUDI to remove coarse PM that typically consists of dust particles prone to bounce off the impaction stages rather than sticking to them. Impaction stages were not coated with an anti-bounce agent in the current study to prevent interference with chemical analysis. Three of the MOUDIs were loaded with pre-baked (48hrs@550^{o}C) aluminum foil substrates that were subsequently used to characterize the PM carbonaceous content. The other three MOUDIs were loaded with clean Teflon membrane substrates that were used to characterize the water-soluble ion and trace metal content of the airborne PM. The elemental carbon (EC) and organic carbon (OC) concentrations from each sample were determined by thermo-optical analysis following the NIOSH temperature protocol (Herner et al. 2005). Water-soluble ions were quantified with Ion Chromatography (IC) analysis and trace elements were quantified with inductively coupled Plasma Mass Spectrometry (ICP-MS) analysis (Herner et al. 2005).

Platelet preparation: As prior published data suggest, platelets might play a critical role in the onset of a pro-coagulant phenotype, which could be a prelude to cardiovascular problems associated with air pollution. Blood samples were collected from the inferior vena cava of sodium pentobarbital anesthetized mice, under institutionally approved protocols, into acid citrate dextrose (ACD) coated syringes with a ratio of 1:10 ACD:blood. Whole blood was incubated at 37^{o}C for 30 min prior to use, with the addition of 10μg/ml PGE_1 (final). Platelet rich plasma (PRP) was prepared by differential centrifugation (660g for 5 min.) and an equal volume of Tyrode's HEPES (12mM $NaHCO_3$, 138mM NaCl, 2.9mM KCl, 10mM HEPES, 10μg/ml PGE_1, pH 7.2) (Norris et al. 2006) mixed with the remaining whole blood and centrifuged at 600g (6 min) at 25^{o}C. PRP resulting from these two spins was combined prior to subsequent processing. It was then centrifuged at 600g for 5 min, the supernatant removed, and the platelet pellet resuspended in Tyrode's HEPES buffer at 1 X 10^8/ml for fibrinogen binding assays. $CaCl_2$ was added to platelets in five equal aliquots at 5 min intervals from a 10mM stock in Tyrode's HEPES to achieve final concentrations of 2mM $CaCl_2$ and 5 X 10^7/ml. All cell counts were determined using an automated blood counter (ActDiff, Beckman-Coulter, Miami FL).

Fibrinogen binding assay: Washed platelets, prepared as noted above, were incubated with Alexa 488-labeled fibrinogen (3μg/ml) for one minute at room temperature prior to activation. Platelets were activated with 20μM ADP and evaluated by flow cytometry after incubation for 30min at 37^{o}C. Alexa 488-labeled fibrinogen binds platelets proportionally to its fluorescence intensity and was used to quantify the extent of fibrinogen binding to platelets. All data were collected with a Beckman-Coulter FC500 flow cytometer. Forward and side scatter voltages were set to detect machine noise, which was removed during subsequent analyses. The FL1 detector was set to 500V to prevent saturation of the detector by emission from the Alexa 488-labeled fibrinogen. Platelet aggregation was determined by the sample light scatter properties. In addition to ADP activated platelets, fibrinogen binding to unstimulated platelets was determined for each sample. Ten thousand fluorescent events were recorded for each condition.

Platelet alpha granule proteins and integrins: Mouse platelet activation was analyzed in whole blood by flow cytometry using three monoclonal anti-mouse antibodies. The major platelet integrin α2b-β3a was evaluated with a biotin-conjugated monoclonal antibody to the α2b subunit (CD41) (BD Pharmingen), followed by Streptavidin Alexa 633. Alpha granule secretion was evaluated with a FITC-conjugated monoclonal antibody to P-selectin (CD62P) (BD Pharmingen), and lysosomal granule secretion was evaluated with a PE-conjugated monoclonal antibody to lysosomal associated protein 1 (LAMP-1)(CD107a) (eBioscience). An isotype control was used for each antibody. Three populations of platelets were examined for each animal, a resting (unstimulated) sample, an ADP (10μM) stimulated sample and a sample stimulated with 0.1U/ml thrombin. After stimulation of whole blood, platelets were labeled with the preceding antibodies for one hour and fixed in 1% (final) paraformaldehyde prior to analysis by flow cytometry. Platelets were defined by forward (FSC), and side scatter (SSC) characteristics and ten thousand events were collected within the platelet gate for each animal and each condition. Platelet-derived (CD41positive) membrane microparticles were identified based on FSC with the x-axis threshold set at 10^{1} representing the lower FSC boundary for unstimulated platelets.

Cytokine assays: Blood samples were collected from the inferior vena cava of sodium pentobarbital anesthetized mice under institutionally approve protocols, and serum was prepared. Cytokine assays were carried out using a commercial immuno-bead based analytical system and mouse specific assay kits (Bioplex, Biorad Life Science, Hercules CA). Activities assayed included Eotaxin, Fibroblast Growth Factor (FGF), Granulocyte Colony Stimulating Factor (G-CSF), Granulocyte Monocyte Colony Stimulating Factor (GM-CSF), Interferon-γ(IFN-γ),Interleukin-1α(IL-1α Interleukin-1β (IL-1β)Interleukin-2 (IL-2), Interleukin-3 (IL-3), Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interleukin-6 (IL-6), Interleukin-9 (IL-9), Interleukin-10 (IL-10), Interleukin-12 protein 40 (IL-12p40), Interleukin-12 protein 70 (IL-12p70), Interleukin-13 (IL-13), Interleukin-15 (IL-15),

Interleukin-17 (IL-17), Interleukin-18 (IL-18), Keratinocyte chemoattractant (KC), Leukemia Inhibitory Factor (LIF), Monocyte Chemotaxtic Protein-1 (MCP-1), Monocyte Colony Stimulating Factor (M-CSF), Monokine Induced by Gamma Interferon (MIG), Macrophage Inflammatory Protein-1α(MIP-1α) Vascular endothelial growth factor (VEGF), Macrophage Inflammatory Protein-1β (MIP-1β)Macrophage Inflammatory Protein-2 (MIP-2), Platelet Derived Growth Factor (PDGF), Regulated Upon Activation, Normally T-Expressed, and presumably Secreted (RANTES) and Tumor Necrosis factor-α (TNFα). All samples were evaluated for all of the cytokines noted above. Data is presented only on cytokines which were statistically significantly different between the study groups.

Lung Preparation: A tracheal cannula was inserted and secured by ligation with suture material. The lungs were removed from the thorax. The left lung lobe was ligated at the mainstem bronchus and removed. The remaining right lung lobes were inflated with 10% neutral buffered formalin at 20 cm H_2O pressure. The left lung lobe was inflated with cyosectioning matrix (50% OCT: PBS) and three transverse sections flash frozen in 100% OCT by immersion in liquid nitrogen cooled isobutane. These three sections were cryosectioned for use in laser capture microdissection experiments. A fourth section of the left lung was flash frozen in liquid nitrogen for cytokine analysis. The formalin instilled right lung lobes were fixed overnight and transverse sections of each embedded in paraffin using standard histotechnology approaches and an automated processor. These blocks were then used to prepare standard hematoxylin and eosin stain slides as well as immunohistochemical staining.

Laser Capture Microdissection: To determine whether there were specific changes in mRNA expression related to either parenchymal, airway or blood vessels, we undertook laser capture microdissection, which allowed us to isolate these three portions of the lung using the following methodology. Lungs from experimental mice were perfused with 50% OCT in saline and flash frozen in a labeled plastic cassette. 15 µm sections were cut using a cryostat, mounted on a glass slide coated with a thin layer of polyethylene naphthalate foil (Leica), and preserved with RNAlater ICE (Ambion) at -20℃ until laser capture microdissection (LCM). LCM was performed at the UC Davis Center for Health and the Environment using a Leica LMD6000 system equipped with an ultraviolet laser and an upright microscope with moving prisms to guide the laser over a stationary sample. Specific tissue regions including large airways, blood vessels (both venous and arterial), and surrounding parenchyma were microdissected and sorted into 3 different microfuge tubes. Total RNA was immediately isolated from pooled samples from each tissue type using an RNeasy Micro Kit (Qiagen), including a DNA digestion step, as described by the manufacturer. Total RNA was quantified and mRNA was reverse transcribed into cDNA using a Superscript III First Strand Synthesis System (Invitrogen). TaqMan assays (Applied Biosystems) specific for genes of interest

were used to quantify gene expression changes using qRT-PCR according to the following amplification parameters: initial denaturation for 10 min at 95℃, followed by 40 cycles at 95℃ for 15 s (melting) and 60℃ for 1 min (annealing and extension). Transcript levels were measured using the ABI Prism 7700 system (Applied Biosystems), and normalized to beta-2 microglobulin (B2M) expression levels for the comparative CT method for relative quantification. Target genes were selected based on *in vitro* experiments with collected particulate matter (unpublished) and previous studies (Wilson et al., 2010).

Whole lung gene array RNA extraction and synthesis of biotin-labeled RNA: Lung tissue (~100 mg) from each mouse was homogenized using TRIzol® Reagent (Invitrogen, Carlsbad, CA) followed by RNeasy Mini Kit (Qiagen, Valencia, CA) including the DNA digestion step as described by the manufacturer. Microarray experiments were performed with pooled RNA isolates from each mouse lung from either air exposed (control) or CAPs exposed group (n = 4, samples were pooled). Confirmatory RT-PCR analysis was done on individual aliquots of RNA from each replicate mouse. A 9 g aliquot of total RNA from each pooled sample was reverse transcribed, followed by 2nd strand synthesis, cRNA amplification in the presence of biotin-labeled nucleotides, and fragmentation as described in the Affymetrix One Cycle Sample Preparation protocol (Affymetrtix, Santa Clara, CA) as previously described (Aung et al 2009). The fragmented, biotin labeled cRNA samples were hybridized to Mouse Genome 430A 2.0 comprised of over 22,600 probe sets representing over 14,500 well-substantiated mouse genes (Affymetrix, Santa Clara, CA). The hybridization, washing, labeling and scanning of the GeneChips was performed as described in the Affymetrix protocols by the Microarray Core Facility in the UC Davis Genome and Biomedical Sciences Facility.

The data were analyzed by GeneChip Operating System (GCOS) 1.4 (Affymetrix, Santa Clara, CA). The upper limit of p value for statistically reliable detection of an mRNA transcript was 0.05 (except for “batch analysis”, see below), independent of its signal intensity (usually >10), because the detected mRNA can be confirmed by an independent method such as quantitative RT-PCR. The p value for detection of mRNAs discussed here ranged from 0.0001 to 0.05 (except for “batch analysis”, see below) and the signal intensity ranged from 10-10000 units; the signal intensities approximate the abundance of mRNAs.

Lists of CAPs exposure sensitive genes were obtained by using the “batch analysis” function in GCOS 1.4. Data for all of the 22,692 probe sets from CAPs exposed mouse lung were compared with those from the filtered air exposed mouse lung. The lists of sensitive genes were then sorted to satisfy three requirements: (a) the mRNA was detectable with a p value of ≤0.05 in at least one of the two samples being compared, (b) the fold-change was ≥2, and (c), the mRNA had an annotation that suggested either a known or a predicted function. The lower limit of 2-fold change was selected and

selected gene expression was confirmed by quantitative RT-PCR. This may be particularly desirable in microarray experiments performed on pooled RNA samples, an analytical strategy that results in loss of statistical information. Affymetrix GCOS software was used to facilitate visualization and interpretation of GeneChip Data.

Validation of changes in mRNA expression by quantitative RT-PCR (qRT-PCR): Since GeneChip analysis was done on pooled samples from each experimental group, and confirming qRT-PCR analyses were performed on individual aliquots of total RNA samples from each treatment replicate. The purpose of these analyses was to evaluate the reliability of GeneChip data and develop statistical data to validate the changes suggested by the GeneChip assay of pooled RNA samples. An aliquot equivalent to 5 µg of total RNA extracted from each sample was reverse-transcribed to obtain cDNA using SuperScript III First Strand kit (Invitrogen, Carlsbad, CA). Real-time polymerase chain reaction (RT-PCR) with SYBR as fluorescent reporter was used to quantify the expression of selected genes identified by GeneChip analysis. Specific primers (Table 1) were designed with Primer Express 1.0 software (Applied Biosystems) using the gene sequences obtained from Affymetrix Probeset IDs. Reactions were carried out in 384 well optical plates containing 25 ng RNA in each well. The quantity of applied RNA was normalized by simultaneously amplifying cDNA samples with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific primers. The transcript levels were measured by real-time RT-PCR using the ABI PRISM 7700 Sequence detection system (PE Applied Biosystems, Foster City, CA). A comparative CT method (Livak et al. 2001) was used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments (Applied Biosystems User Bulletin No.2 (P/N4303859). The threshold cycle, Ct, which correlates inversely with the target mRNA levels, was measured as the cycle number at which the SYBR Green emission increases above a preset threshold level. The specific mRNA transcripts were expressed as fold difference in the expression of the specific mRNAs in RNA samples from the CAPs exposed lung compared to those from the air exposed lung of mice.

Immunocytochemistry: Sections of lung were de-paraffinized in graded alcohols and rehydrated in water. Sections were immersed in a solution of 0.05T Tween-20 with 50mM Tris and 150mM NaCl (pH=8.5) and microwaved for 75 min and allowed to cool to room temperature prior to staining. Endogenous peroxidases were blocked and non-specific binding blocked by the addition of 5% bovine serum albumin. Sections were washed thrice and incubated in primary antibodies (CD14 (Serotec 1:500), E-selectin (R&D 1:200), I-CAM-1(R&D 1:200) or isotype controls at the same dilutions as primary antibodies) for one hour at room temperature. Sections were washed thrice and incubated with secondary antibodies conjugated to horse radish peroxidase polymers, followed by a derivitized diaminobenzidine chromogen. All sections were counter-stained with hematoxylin. Once labeling of slides was complete the slides were

dehydrated through graded ethanols to xylene, coverslipped and allowed to dry. Sections were viewed on a bright field microscope.

Statistical Analysis: For all assays, comparisons between control and CAPs exposed animals were evaluated by Student's T-test with a significance level of $p<0.05$.

Quality Control of data/equipment:

Quality Assurance (QA): The University of California, Davis is committed to producing quality environmental exposure data and has implemented a QA program for all field sampling, laboratory analysis, and data analysis efforts. A key function of this program was to document the methods used to collect and analyze the data so that the data produced are scientifically valid and defensible.

Data collection activities: Data quality objectives and acceptance criteria, characterized in terms of accuracy, precision, detection limits (LODs), completeness, and representivity, were set based on the intended use (described below). Our quality system complies with ANSI/ASQC: "Specifications and Guidelines for Quality Systems for Environmental Data Collection and Environmental Data Collection and Environmental Technology Programs"

Handling of PM samples: Teflon filters were equilibrated inside the weighing room with controlled temperature and humidity before use. After being neutralized by Polonium-210 sources, the filters were weighed by the microbalance at up to 1μg resolution. The Mettler 5 Microbalance was checked in advance by using a 100.000-mg stainless steel standard and 47mm blank filter. The microbalance reading after a 30-second stabilization time was recorded in the logbook together with temperature and relative humidity value at that time. Finally, weighed filters were transferred to labeled petri dishes using sterile tweezers. Filter handling of quartz and glass fiber filters was similar to Teflon. The filters were taken from the vacuum dessicator and refrigerator, respectively, where they were stored and placed into the filter holders.

Particle Concentration Measurement: Particle number distributions were measured as a function of size using a scanning mobility particle sizer (SMPS) (TSI). The SMPS measures particle number distributions in the size range from 15–800 nm particle diameter with a time resolution as fast as 60 sec. These measurements were performed using the TSI Condensation Particle Counter (CPC, Model 3022, TSI Inc., St. Paul, MN). The uncertainty in the concentration measurements was ± 2%.

Carbon Analysis: The carbon concentration was determined by thermo-analysis. From each filter, an aliquot of approximately 0.2 cm^2 was placed in a platinum boat containing MnO_2. The sample is acidified with a dilution of HCl and heated to 115 °C to remove the water and CO_2 (from sample carbonates). The boat then was advanced into a dual zone

furnace where MnO_2 oxidized OC in the sample at 550 °C and EC at 850° C. The CO_2 formed is converted to CH_4 for detection by a Flame Ionization Detector (FID). The analytical method is described in detail by Fung et al. (1990).

PAH analysis: PAH analysis was done by GC/MS using core facilities and personnel in the Air Quality Research Center as previously described (Kado et al. 2005).

Iron Analysis: Iron analysis was done by XRF using core facilities and personnel in the Air Quality Research Center as previously described (Zhou et al. 2003).

Histological Samples: All tissues for morphological assessment were processed under rigid conditions. A record of each embedment procedure was maintained as well as acquisition numbers for each tissue specimen. These numbers were recorded in the acquisition number log book as a permanent record.

Biochemical assays: Biochemical assays were validated for each experiment. All biochemical assays were performed in triplicate to assure accuracy and reproducibility. Laboratory notebooks were meticulously maintained for each experiment. In addition, standard quality assurance and quality control procedures as designated by the American Society for Testing Materials and the American Public Health Association were practiced in our laboratories.

Instrument calibration: Standard operating procedures for all instruments, data processing, field, and quality control protocols for all equipment and procedures were documented in written manuals. All instrumentation were calibrated according to fixed schedules, manufacturer's instructions, and most importantly, to the requirements of the analysis as dictated by the standard operating procedure (SOP). The laboratory balances were maintained and calibrated on a continual basis. External calibrations and routine maintenance were performed annually by an independent contractor. Audits verified that the balances were functioning within the manufacturer's specifications. Part of the AQRC exposure core responsibility included periodic maintenance of the aerosol generating system, as well as technical supervision during the exposures to ensure proper operation. In the beginning and at the end of each exposure, the flow rate at the outlet of each system (i.e., upstream of the exposure chamber) was recorded. Pressure gauges were placed in the major and minor flows of the system to ensure that the flows through each stage were stable and that the flow rates in the major and minor flows reached their appropriate values.

Statistical analyses: We used a statistical analysis that reflects our single factor design. Differences between groups were assessed by one-way analysis of variance (ANOVA) and all pairwise multiple comparison tests (Tukey) were also performed as relevant. All comparisons with $p \leq 0.05$ were considered significant (Glantz, 1992). Statistical

consulting was available from Dr. Neil Willits in the UCD department of statistics under a collaborative agreement with the UCD Air Quality Research Center. At each stage of the analysis, statistical significance was defined as $p<0.05$.

Intended use of the data: Data generated in this study was used to assess and document the effects of ambient fine particles on healthy animals. The information generated in the exposures will be published in the open literature to be used by air-quality managers and environmental health scientists.

Safety: All personnel associated with this proposal attended periodic review courses on laboratory and chemical safety. Environmental Health and Safety personnel made unannounced inspections of our facilities. A description of testing procedures necessary to demonstrate required performance of equipment to be used in the study is available for inspection. Flow cytometers were calibrated daily as per manufacturer's instructions to ensure consistent detection systems.

Table 1: Tissues Examined and Assays Performed

Whole Blood and Platelets	CD41b Flow Cytometry	P-selectin Flow Cytometry	LAMP-1 Flow Cytometry	Fibrinogen Binding Flow Cytometry	Platelet Counts	Complete Blood Counts
Lung	Whole Lung Gene Expression	Cytokine protein expression	Airway gene expression	Parenchyma gene expression	Vessels gene expression	Immunohisto-chemistry Histology
Serum	Cytokine Protein levels					

Results:

For simplicity's sake we will divide up these studies by date and location. We will first describe the results of the three CAPs experiments (Winter 08 Rural Fresno, Summer 08 Urban Fresno and Winter 09 Urban Fresno), followed by the data from the two intra-tracheal experiments using particles from Summer 08 Urban Fresno and Winter 08 Rural Fresno.

Study One: The first study we will describe is the winter 2008 Rural (Fresno) Field Study. Data from the 2008 winter rural CAPs study (Wilson et al. 2010) demonstrated three critical findings. Firstly, the study demonstrated increased platelet numbers in the peripheral whole blood of CAPs exposed animals compared to FA exposed animals. This increase was specific to this CAPs exposure, and was not seen in any of the other studies within this project. It was, however, statistically significant as platelet numbers in control animals were 604 +/- 305 X 103/µl compared with 992 +/- 213 X103/µl from CAPs exposed animals. In all other studies there were no changes in the complete blood counts, or in the types of white blood cells seen in the peripheral blood. The second critical finding was that we saw significant increases in a large number of systemic cytokines, which are shown in Figures 1 and 2. These cytokines included both TH-1 and TH-2 cytokines, as well as cytokines specifically associated with platelet upregulation. Generally, there was an increase in interleukins-6 and -10, macrophage inhibitor protein 1 alpha (MIP-1α) and beta (MIP-1β), granulocyte-monocyte (GM-CSF) colony stimulating factor as well as tumor necrosis factor alpha (TNFα). Interestingly, there were also significant increases in both RANTES and PDGF-bb both of which are stored and released from platelet alpha granules.

Figure 1: Serum cytokine levels: Winter 2008 Fresno study. Data presented as mean +/- SEM, * =p<0.05 using a 2 tailed t test, ** =p<0.05 using a one tailed t-test.

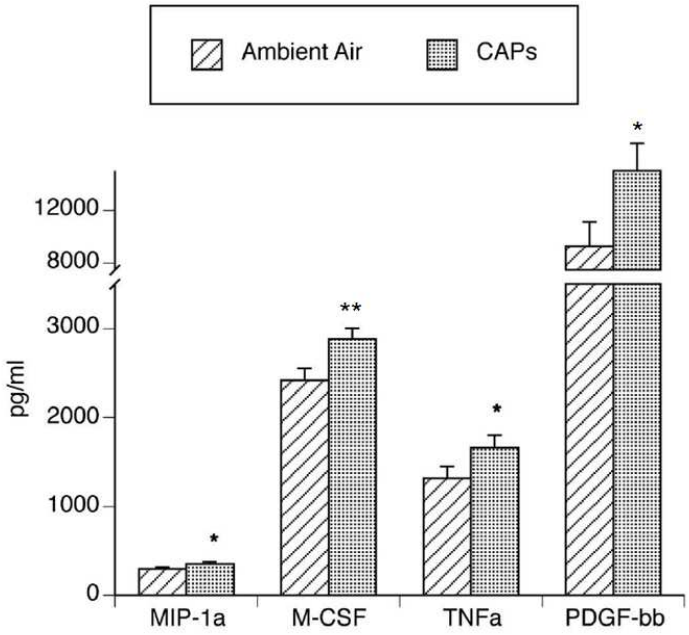
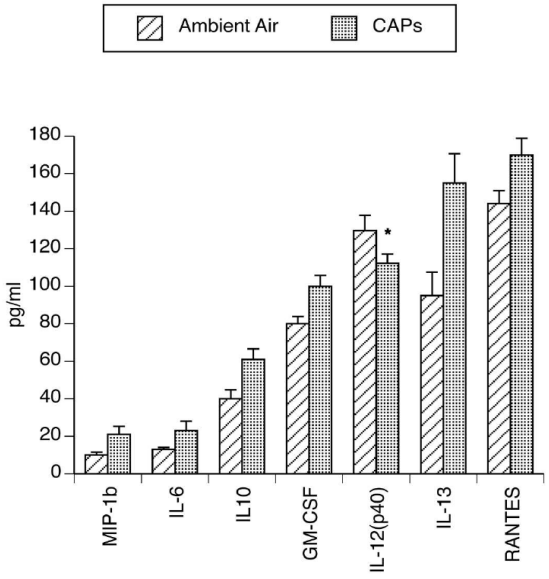


Figure 2: Serum cytokine levels: Winter 2008 Fresno study. Data presented as mean +/- SEM, * =p<0.05 using a 2 tailed t test.



Lastly, there were clear indications of platelet up-regulation as determined by alterations in the platelet phenotype by flow cytometry (Figures 3,) as well as by an increase in platelet microparticles, characterized as small CD41 (integrin α2b) positive events (Figure 3C). Platelet activation was characterized by an increase in the number of platelets which expressed the activation marker, lysosomal associated membrane protein -1 (LAMP-1) on the cell surface (Figure 4) as well as binding of fibrinogen by unstimulated platelets from CAPs animals (Figure 5).

Figure 3: Representative flow cytometric panels of platelets from mice exposed to either ambient air or CAPs. Each panel represents analysis of 10,000 platelets from an individual animal. Panels A and B represent the forward scatter (FSC) vs. side scatter (SSC) dot plots of resting (unstimulated) control (A) and CAPs (B) exposed mouse platelets. A large, well-defined population of platelet aggregates, as indicated by arrows, can be seen in panel B consistent with platelet activation. Platelet microvesicles can be identified in the lower left quadrant of the preparations, particularly those from CAPs exposed animals. (C) When platelets are stimulated with agonist, platelets from CAPs exposed animals have a higher percentage of events in the microparticle gate than do platelets from ambient air exposed animals (n=8, mean + SD, * = p<0.05).

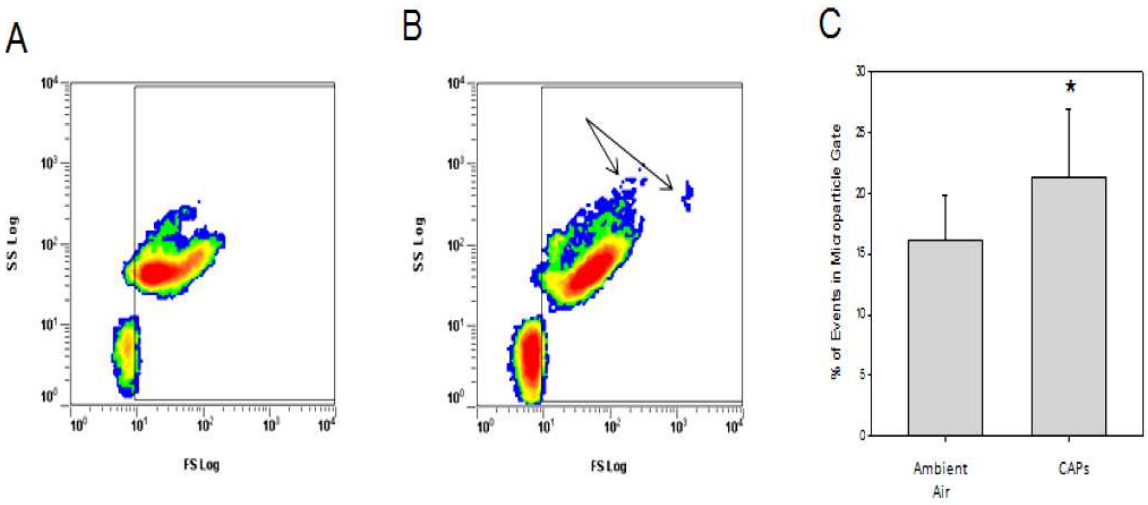


Figure 4: Representative flow cytometric analysis of LAMP-1 expression on the surface of control (A) and CAPs (B) exposed mouse platelets. Ten thousand positive events were analyzed for each sample. The shaded gray area represents the isotype negative control, while the thin line represents the expression of LAMP-1 on the surface of unstimulated (resting) platelets. There is no difference in the expression of LAMP-1 between these two conditions. However, when platelets are exposed to stimulation by thrombin (dashed lines), control platelets show a small response, while a significantly greater number of CAPs exposed platelets express LAMP-1 on their surface (p=0.038). In addition there is a two-fold increase in the mean fluorescence intensity of LAMP-1 expression on thrombin stimulated CAPs platelets when compared with control platelets (n=8, mean + SD, p=0.08).

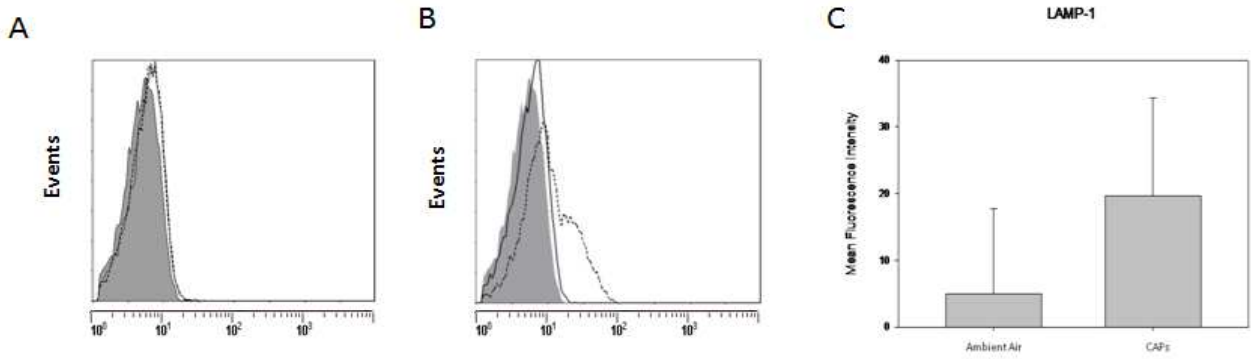
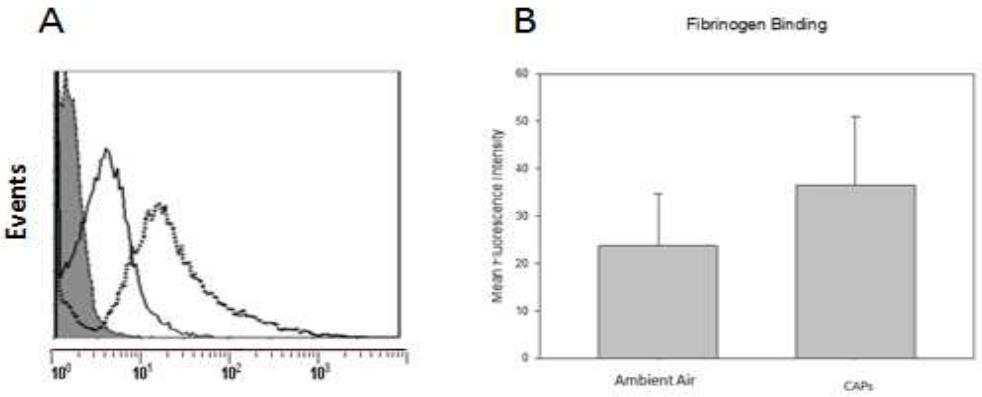
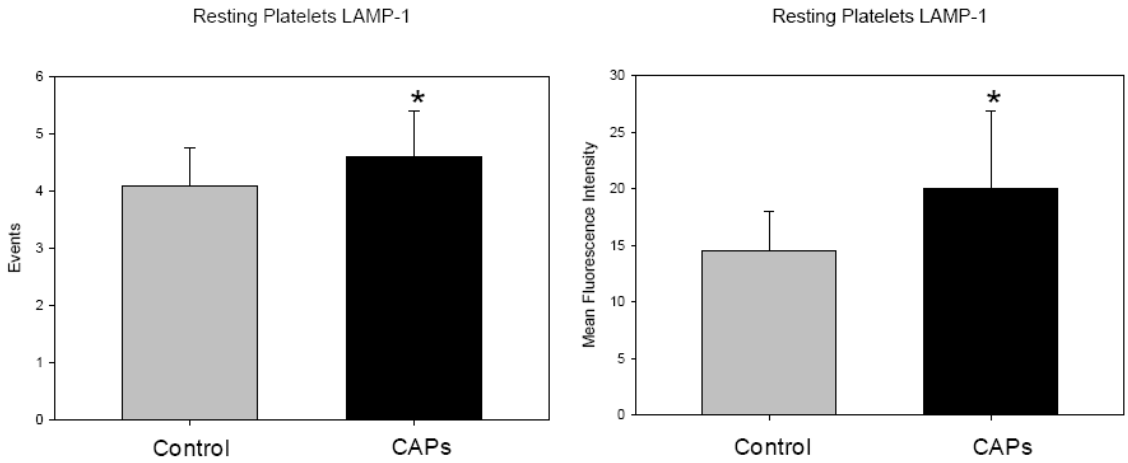


Figure 5: Representative flow cytometric panels from ambient air exposed and CAPs exposed mouse platelets labeled with fibrinogen. Ten thousand positive events are analyzed for each animal studied. The shaded gray area represents the autofluorescence control in both panels. Panel A depicts histograms of fluorescence intensity of labeled fibrinogen binding for resting platelets from mice exposed to by filtered air (solid line) or CAPs (dashed line). Panel B shows the difference in mean fluorescence intensity of fibrinogen binding between control and CAPs exposed platelets (n=8, mean, + SD, p=0.06).



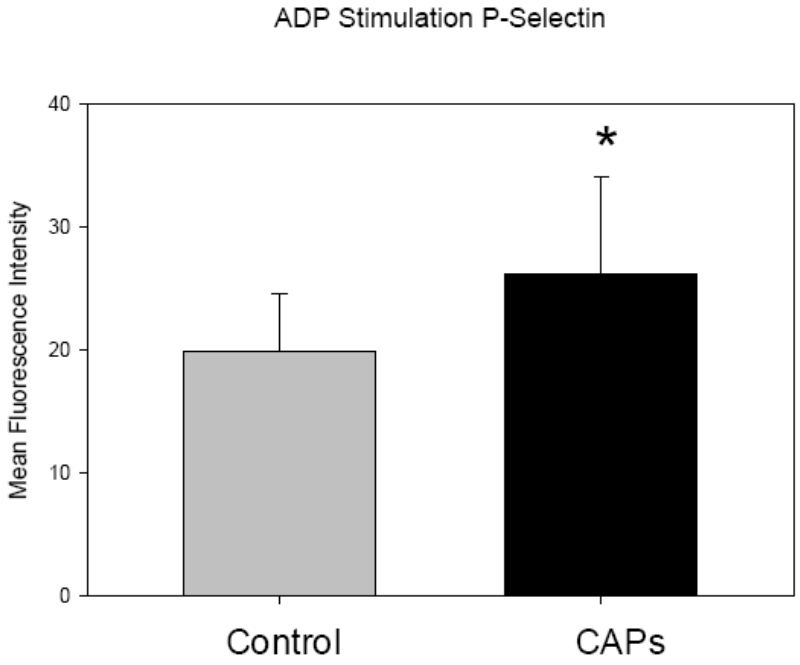
Study Two: The second study was the summer 2008 Field Study – an urban Fresno CAPs study. Our analysis of the serum cytokines in this field study demonstrated that in CAPs exposed mice, there were virtually no differences in serum cytokines compared to control mice. However, for one cytokine – IL12 p40, there was a trend toward a 26% greater increase in expression compared to the filtered air exposed mice (p=0.08, using a two-tailed t-test). Similarly to the winter 2008 rural field study, platelets were again seen to be activated. Resting platelets, those not treated with agonist, showed activation through the expression of LAMP-1. There were increased numbers of positive platelets in the LAMP-1 gate and the mean fluorescence intensity of those platelets was significantly greater than platelets from filtered air exposed animals. (Figure 6)

Figure 6: Left hand panel demonstrates the differences in the number of LAMP-1 positive events between control and CAPs exposed platelets. The right hand panel demonstrates that within the LAMP-1 positive population, CAPs exposed platelets had greater mean fluorescence intensity. (n=6, mean + SD, *indicates p<0.05)



When platelets were treated with ADP, a physiological agonist, platelets from CAPs exposed animals had significantly greater expression of the alpha granule protein P-selectin on their cell surface than filtered air exposed animals. (Figure 7).

Figure 7: ADP stimulation of platelets demonstrates that CAPs exposed platelets have a greater response to ADP as indicated by an increased P-selectin exposure. (n=6, mean + SD, * =p<0.05.



Unlike the winter rural study, there were no significant hematologic changes in platelets, red blood cells or white blood cells. Laser capture microdissection of pulmonary vessels and airways followed by mRNA expression analysis demonstrated that in the vessels there was an increase in E-selectin as well as in TNF-α, suggesting increased inflammation in the pulmonary vasculature. In addition, there was a slightly increased expression of E-selectin in the airway epithelium as well. (Figure 8)

Figure 8A: Laser Capture Microdisssection of Summer 2008 Airways

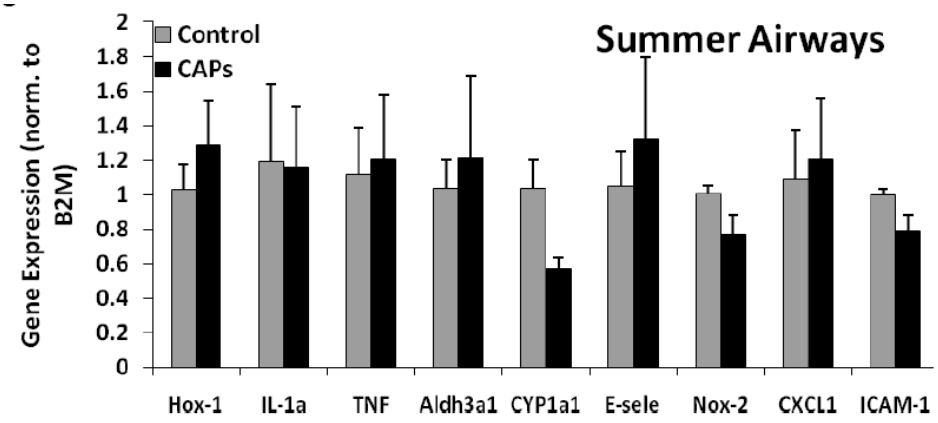
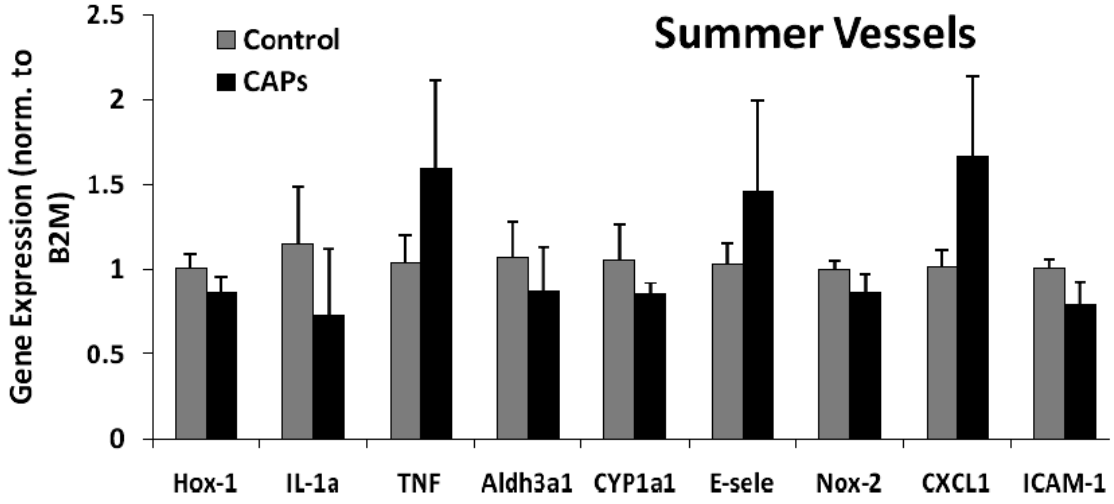


Figure 8B: Laser capture microdissection of Summer 2008 Vasculature



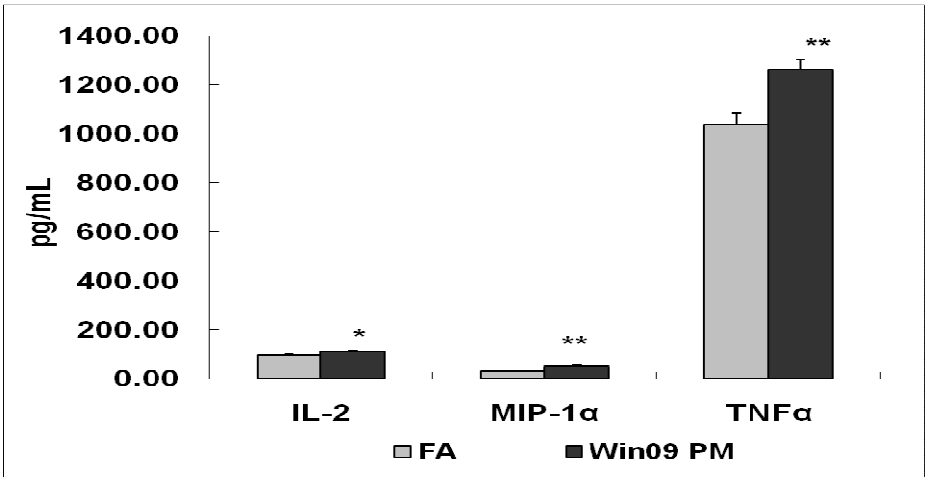
Blinded histologic examination of paraffin embedded sections did not reveal any degree of inflammation in the lung overall. However, gene micro-array analysis of whole lung showed several 2 fold upregulated genes, erythroid differentiation regulator 1 (Erdr1) and myelin basic protein (Mbp) and 4 down-regulated genes: phosphoenolpyruvate carboxykinase 1, cytosolic (Pck1, 2.1 fold decrease) Immunoglobulin kappa chain variable 1 (Igk-V1 2.3 fold decrease) angiopoietin-like 4 (Angptl4 2.6 fold decrease) and immunoglobulin heavy chain complex (Igh 4.9 fold decrease)(Table 2).

Table 2: Gene Microarray Results from 2008 Summer Urban Study

Gene Title	Gene Symbol	Representative Public ID	Fold Change
predicted gene	629242	BC002257	2.5
myelin basic protein	Mbp	AI323506	2.3
erythroid differentiation regulator 1	Erdr1	AJ007909	2.1
predicted gene	100039054	NM_031188	2.1
actin, beta	Actb	NM_007393	2.0
phosphoenolpyruvate carboxykinase 1, cytosolic	Pck1	AW106963	-2.1
immunoglobulin kappa chain variable 1 (V1)	Igk-V1	U29768	-2.3
angiopoietin-like 4	Angptl4	NM_020581	-2.6
immunoglobulin heavy chain complex	Igh	BC018365	-4.9

Study Three: The third study was the winter 2009 Fresno Urban CAPs Field Study. Animals in this study were either exposed to CAPs as indicated above, or to filtered air. Endpoints for this study were: complete blood counts, platelet activation, serum cytokines, whole lung gene micro-array and lung laser capture and gene expression. Similar to our data from the summer 2008 Fresno urban study, there were limited changes in serum cytokines for this winter urban study. However, there were 4 cytokines which were statistically significantly up-regulated. They were: IL-2 ($p=0.035$), IL-4 ($p=0.016$), MCP-1 ($p=0.05$), MIP-1α ($p=0.0005$), and TNF-α ($p=0.04$) (Figure 9).

Figure 9: Serum cytokines (mean +/- SEM) from Winter 09 Urban CAPs study. Please note that MCP-1 is not shown on the graph as it was conducted with a 1-tailed T-test, indicating limited significance. * =p<0.05, ** =p<0.01



As was the case for the summer 2008 urban CAPs studies, there were no significant changes in the numbers and parameters for red blood cells, platelets, and white blood cells. Platelet activation was also observed for this study. In addition to examining platelet response to agonist by evaluation of LAMP-1, P-selectin and the integrin α2bβ3a, we also examined the degree of fibrinogen binding in response to the physiological agonist ADP. Platelets from CAPs exposed animals showed a trend ($p=0.07$) toward increased fibrinogen binding, as demonstrated by a 0.76 fold increase compared to FA exposed animals. Flow cytometric analysis of platelet activation markers demonstrated that platelets from CAPs exposed animals showed a greater response to thrombin both by an increase in mean fluorescence intensity of P-selectin ($p=0.013$), as well as by the number of LAMP-1 positive platelets ($p=0.015$). (Figure 10 A, B)

Figure 10A: Platelets from CAPs exposed animals respond to thrombin stimulation by expressing more LAMP-1 on their surface, as indicated by significantly increased mean fluorescence intensity. (n=6, mean + SD)

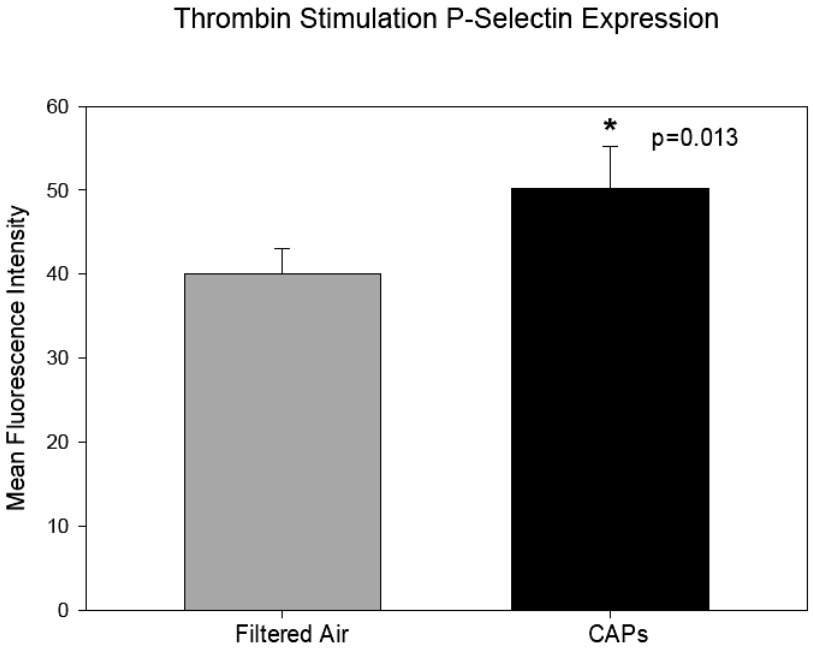
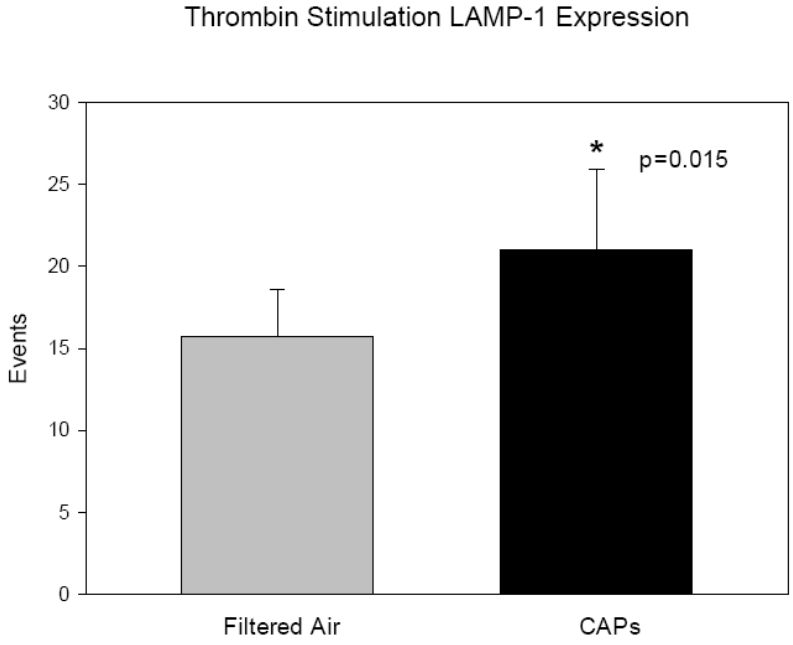


Figure 10B: Greater numbers of platelets from CAPs exposed animals respond to thrombin stimulation than do those from animals exposed to filtered air. (n=6, mean + SD)



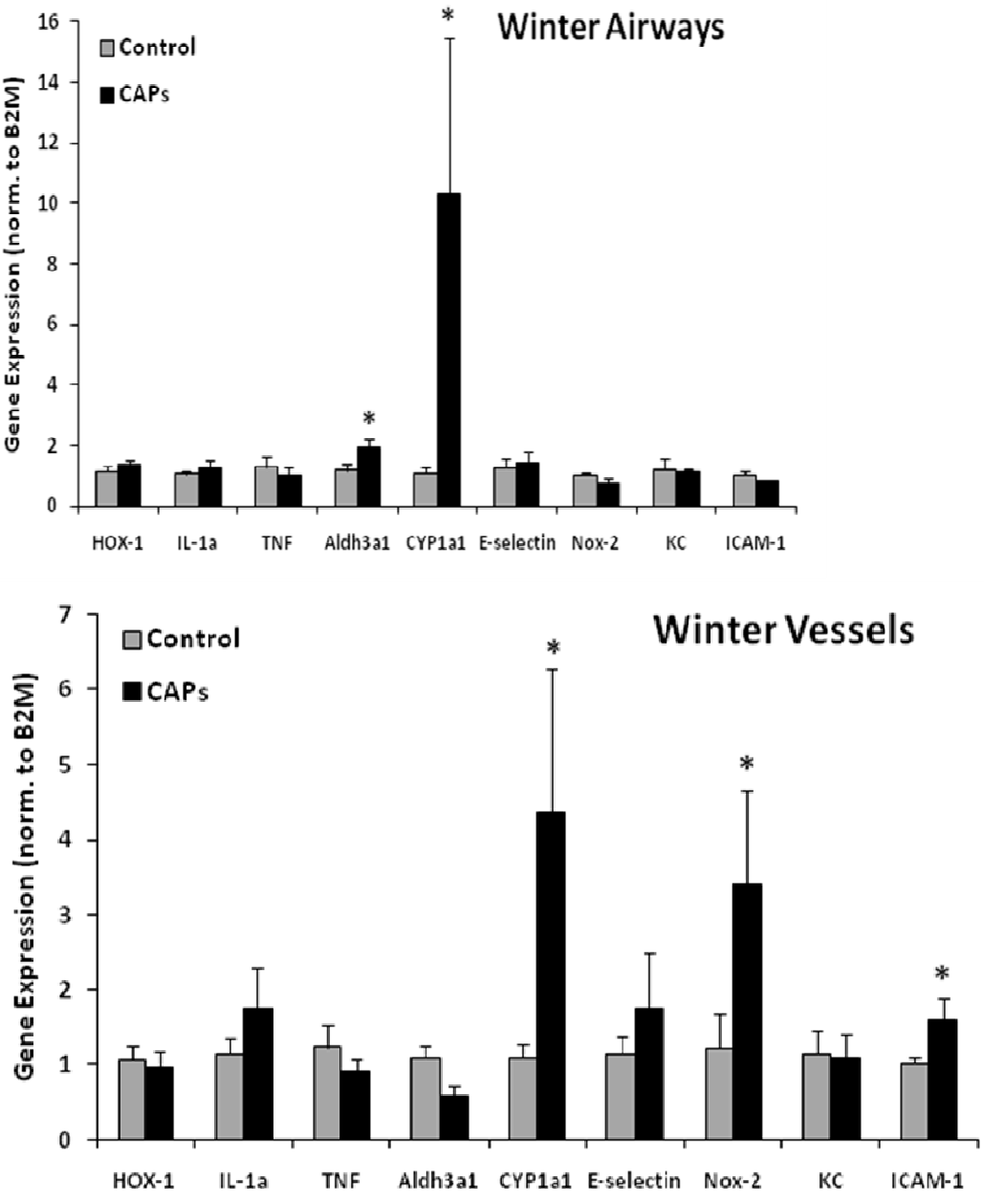
Gene micro-array of whole lung demonstrated 2 fold increases in cardiac muscle myosin light polypeptide and myosin heavy polypeptide. In addition there was a 2 fold increase in parvalbumin, a calcium binding protein. A large number of genes were 2 fold down-regulated including but not limited to: cytochrome P450, heat shock protein 1A, cell death-inducing DNA fragmentation factor. Phosphoenolpyruvate carboxykinase 1 was 4.9 fold down-regulated (Table 3).

Table 3: Gene Microarray Results From 2009 Winter Urban Study

Gene Symbol	Gene ID		Fold Change
myosin, heavy polypeptide 2, skeletal muscle, adult	Myh2	NM_001039545	2.6
parvalbumin	Pvalb	NM_013645	2.1
myosin, light polypeptide 2, regulatory, cardiac, slow	Myl2	NM_010861	2.0
chitinase, acidic	Chia	NM_023186	-2.0
citrate lyase beta like	Clybl	NM_029556	-2.0
cytochrome P450, family 2, subfamily e, polypeptide 1	Cyp2e1	NM_021282	-2.0
erythroid differentiation regulator 1	Erdr1	NM_133362	-2.0
heat shock protein 1B	Hspa1b	NM_010478	-2.0
orosomucoid 1	Orm1	NM_008768	-2.0
transmembrane protein 97	Tmem97	NM_133706	-2.0
zinc finger and BTB domain containing 16	Zbtb16	NM_001033324	-2.0
heat shock protein 1A	Hspa1a	NM_010479	-2.1
predicted gene	100039054	NM_001045550	-2.1
prolyl-tRNA synthetase (mitochondrial)(putative)	Pars2	NM_001083887	-2.1
---	---	---	-2.3
eukaryotic translation initiation factor 2, subunit 3, structural gene Y-linked	Eif2s3y	NM_012011	-2.3
uncoupling protein 1 (mitochondrial, proton carrier)	Ucp1	NM_009463	-2.3
elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3	Elovl3	NM_007703	-2.5
cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	Cidea	NM_007702	-2.6
phosphoenolpyruvate carboxykinase 1, cytosolic	Pck1	NM_011044	-4.9

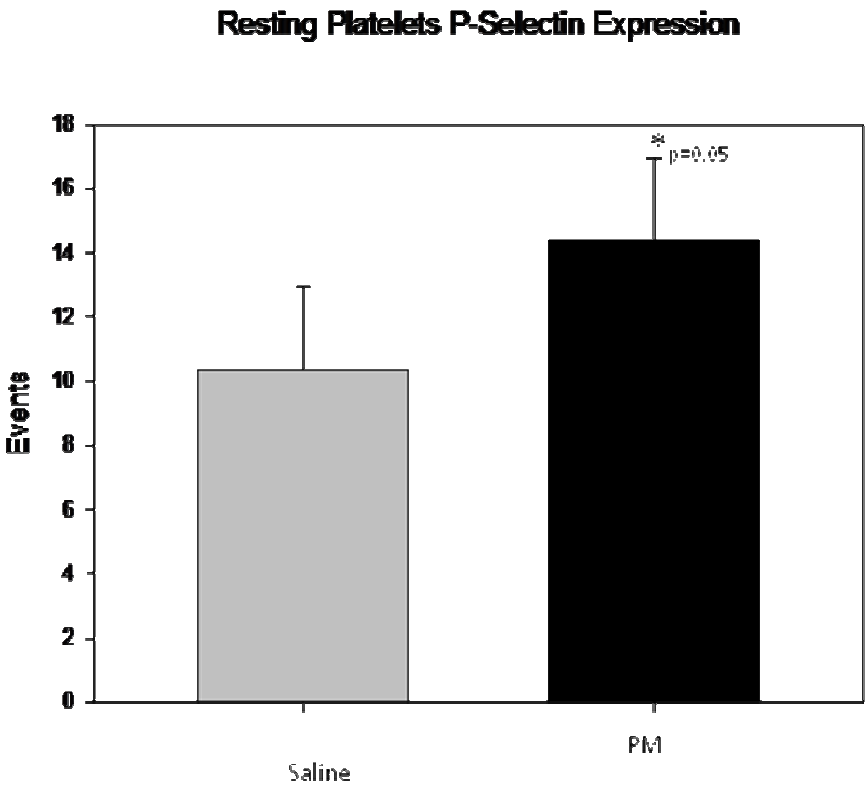
Laser capture data indicated that mRNA expression in pulmonary vessels from CAPs exposed animals showed significant ($p<0.05$) increases in Nox-2, CYP1a1, and ICAM-1, while airways showed increases in CYP1a1 and Aldh3a1 (Figure 11).

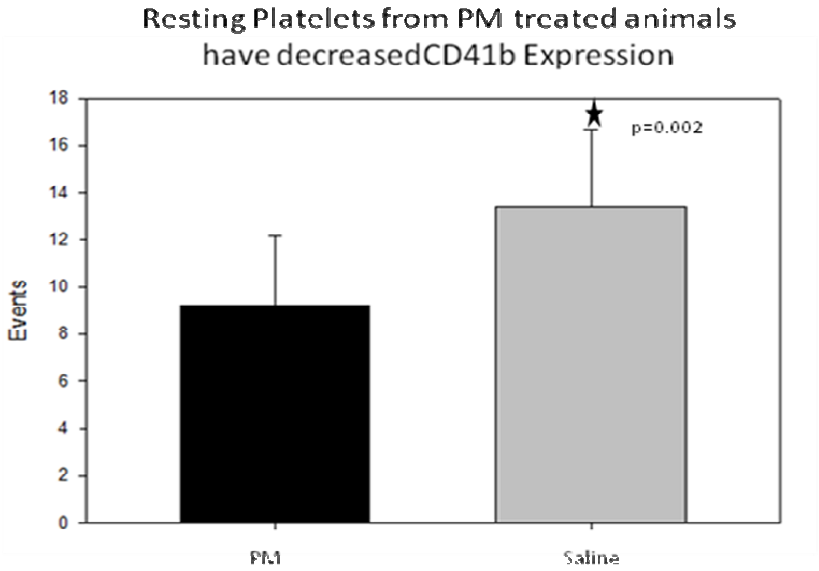
Figure 11: LCM data below examine gene expression in airways (top figure) and vessels (bottom figure). In both cases there is a statistically significant increase in CYP1A1. In addition there is an increase in the PAH response element Aldh3a1 in the airways, while there is an increase in the reactive oxygen species Nox-2 in the vessels. (mean + SEM, * =p<0.05)



Studies Four and Five: The fourth and fifth studies were conducted using particles previously collected during the CAPs studies described above (Summer 2008 Urban Fresno and Winter 2009 Urban Fresno), which were administered via intra-tracheal instillation. Both of these studies showed similar platelet effects, however, there were alsodifferent results from the CAPs studies. The first intra-tracheal study was done with particles collected from urban Fresno in the Summer 2008. Flow cytometric analysis of platelet activation demonstrated that resting platelets (those not yet treated with physiologic agonists) from particle (PM) instilled mice had greater alpha granule secretion as evidenced by increased number of positive platelets in the gate ($p<0.05$). In addition this same population of resting platelets had decreased amounts of CD41 (the integrin a2b subunit, $p=0.002$) on their membranes strongly suggestive of platelet activation. (Figure 12).

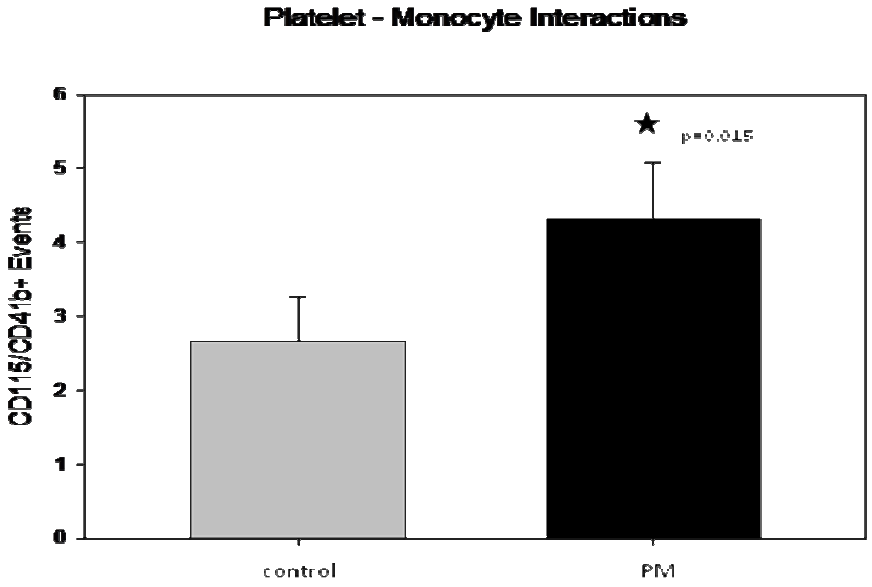
Figure 12: Platelets from CAPs instilled mice express P-selectin, an alpha granule membrane protein on their surface, in the absence of agonist stimulation (top figure). Additionally, the platelets express less CD41b (bottom figure) indicative of platelet activation. (n=6, mean + SD).





We also wished to determine whether treatment platelet activation was associated with increased platelet-monocyte and/or platelet-leukocyte interactions. Flow cytometric analysis of platelet monocyte interactions – as evidenced by labeling both with CD41b (platelet integrin alpha2b) and CD115 (a monocyte specific marker) demonstrated that animals who had received particles showed almost a 2 fold increase in the number of platelet-monocyte interactions compared to control saline treated animals (p=0.015), as shown below in Figure 13.

Figure 13: Platelets (labeled with CD41b) and monocyte (labeled with CD115) aggregates were evaluated in whole blood. Events that were positive for both markers indicated aggregates. PM treated animals showed significantly more platelet-monocyte aggregates than animals treated with saline alone (controls). (n=6, mean + SD)



Evaluation of platelet-leukocyte interactions showed no differences between saline instilled and particle instilled animals. Serum cytokine studies demonstrated that there were two distinct sets of cytokine responses. A group of responses were characterized by decreased serum cytokines relative to saline controls: KC (p=0.013), MIP 1-α (p=0.02), RANTES (p=0.05). TNF-α also showed a downward trend toward significance (p=0.07). (See Figure14A). A second group of cytokines increased in PM instilled animals compared to controls, including FGF (p=0.01), LIF (p=0.01) , M-CSF,(p=0.03) and MIP-2 (p=0.04). (See Figure 14B).

Figure 14A: Serum cytokines decreased in animals instilled with PM compared to saline controls.

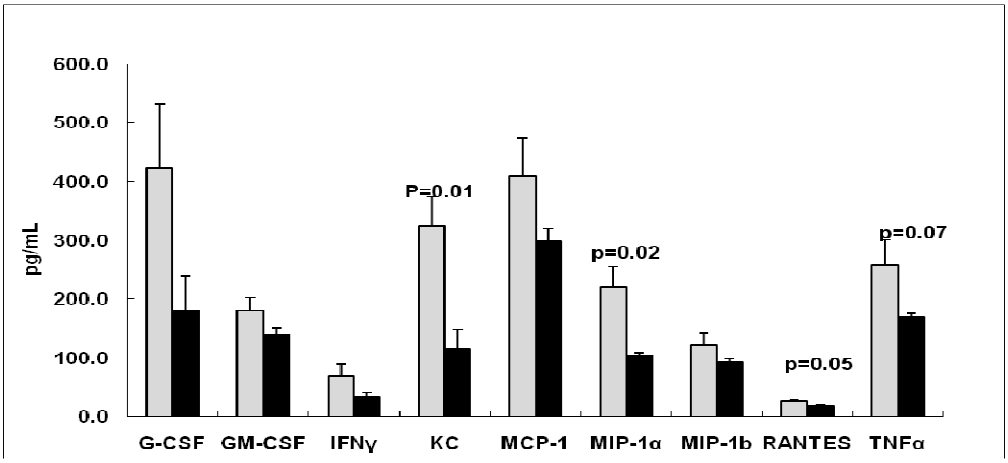
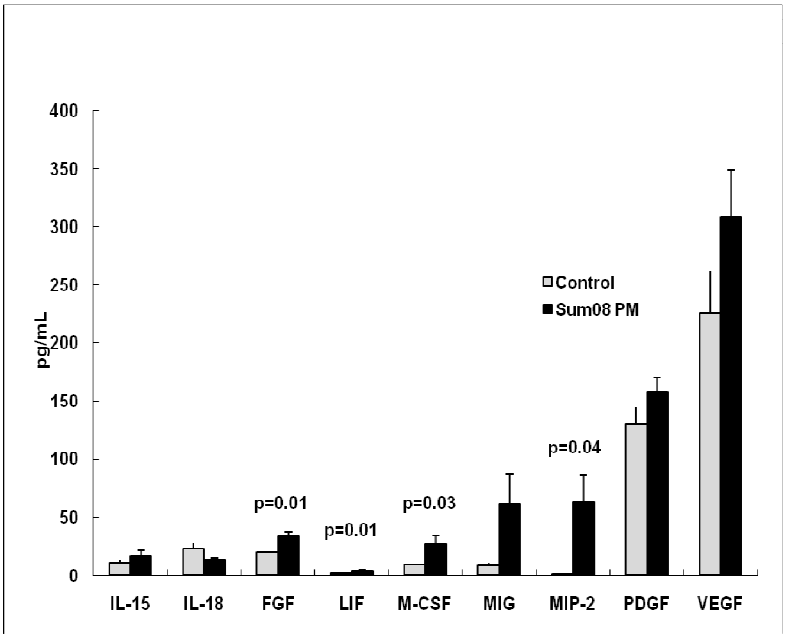
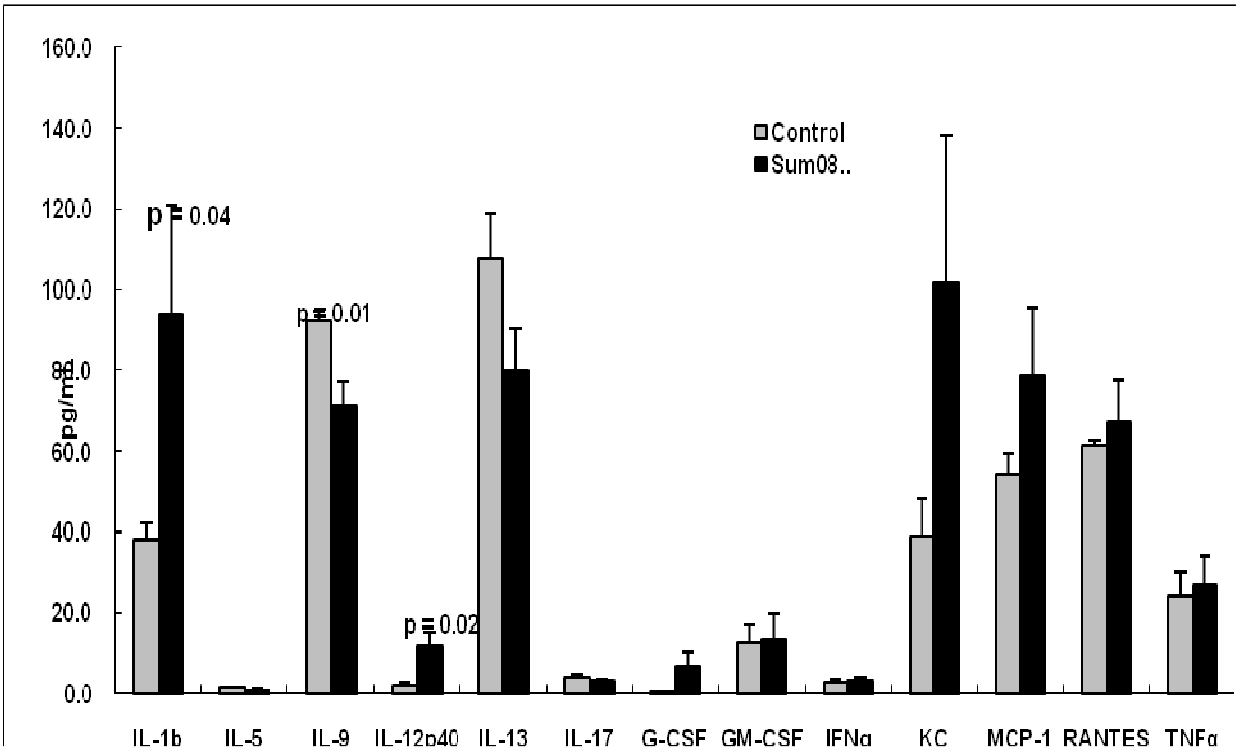


Figure 14B: Serum cytokines increased in animals instilled with PM compared to saline controls.



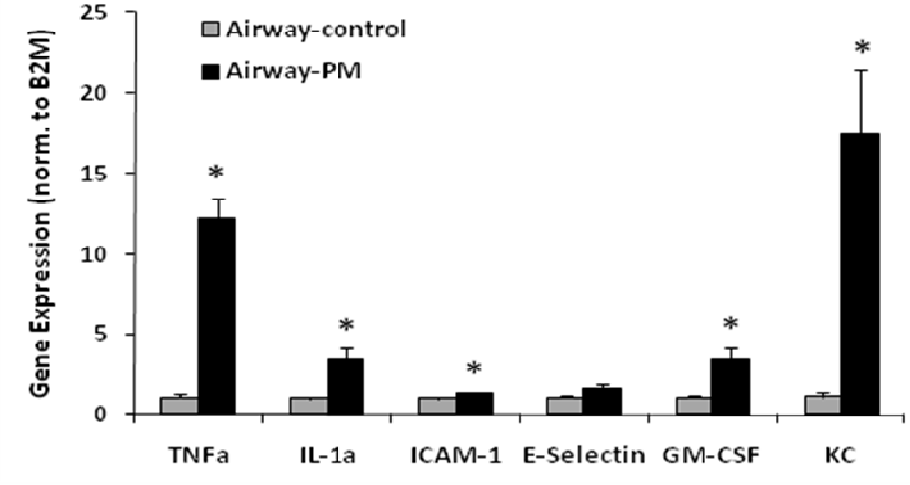
Evaluations of cytokines from preparations of whole lung demonstrated changes in only three cytokines. IL-1b (p=0.04) and IL-12p40 was significantly increased in PM treated animals (p=0.04) while IL-9 was significantly decreased in PM treated animals (p=0.012) (Figure15)

Figure 15: Whole lung cytokines in animals instilled with PM compared to control animals.



Laser capture studies demonstrated statistically significantly elevated mRNA expression in TNFα in airways, parenchyma and pulmonary vessels. mRNA that was also increased in airways included IL-1a, ICAM-1, GM-CSF and KC (IL-8 homologue in the mouse) (Figure16). Additionally, several genes were down-regulated, including tissue factor, CYP1a1 and Nox-4. (Figure16).

Figure 16: mRNA expression in the airways of mice instilled with PM compared to controls. *=p<0.05



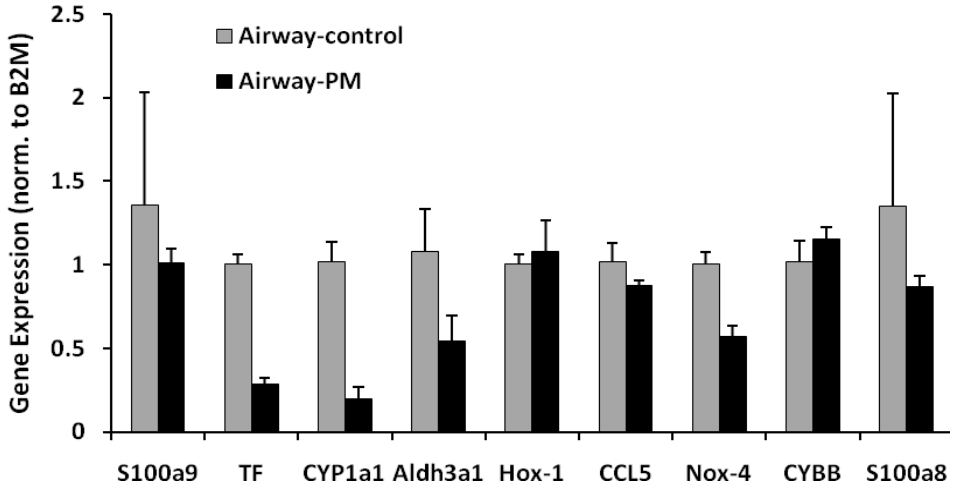
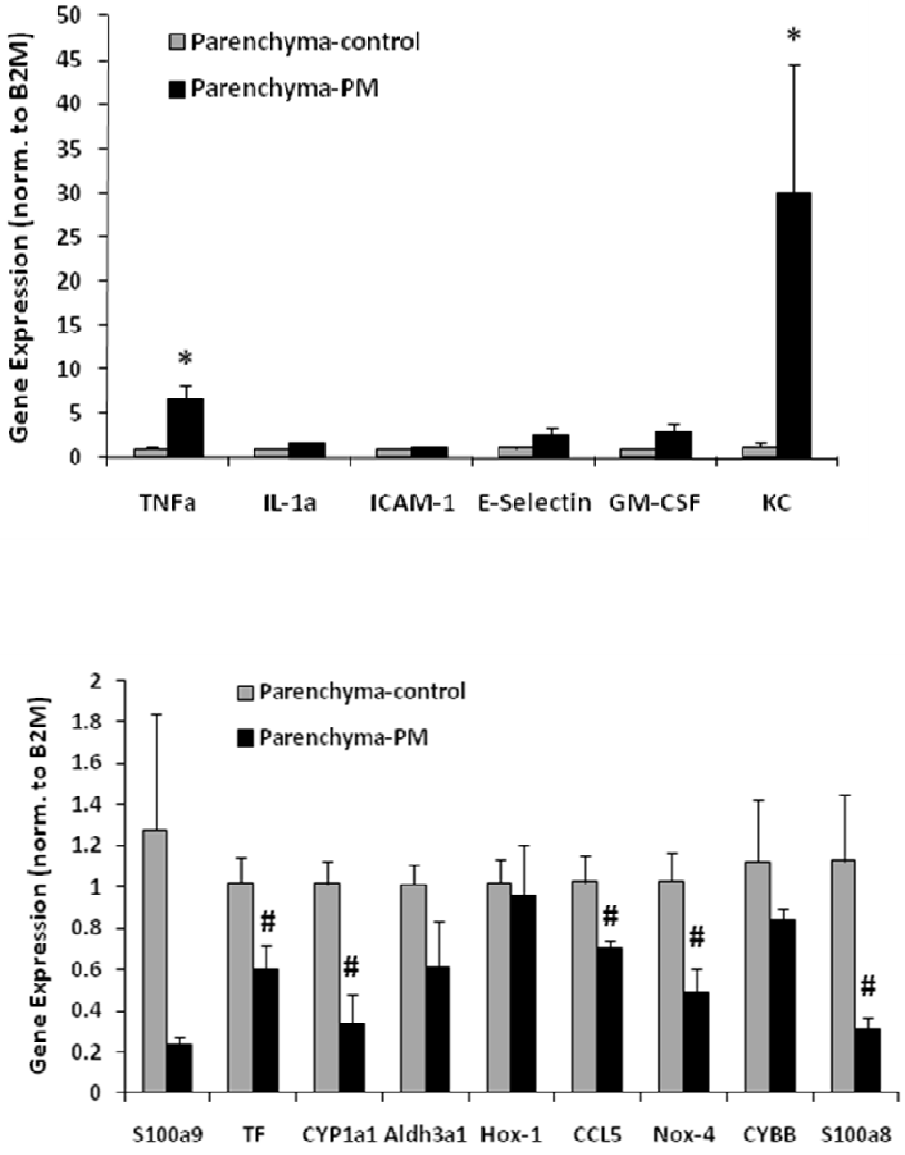
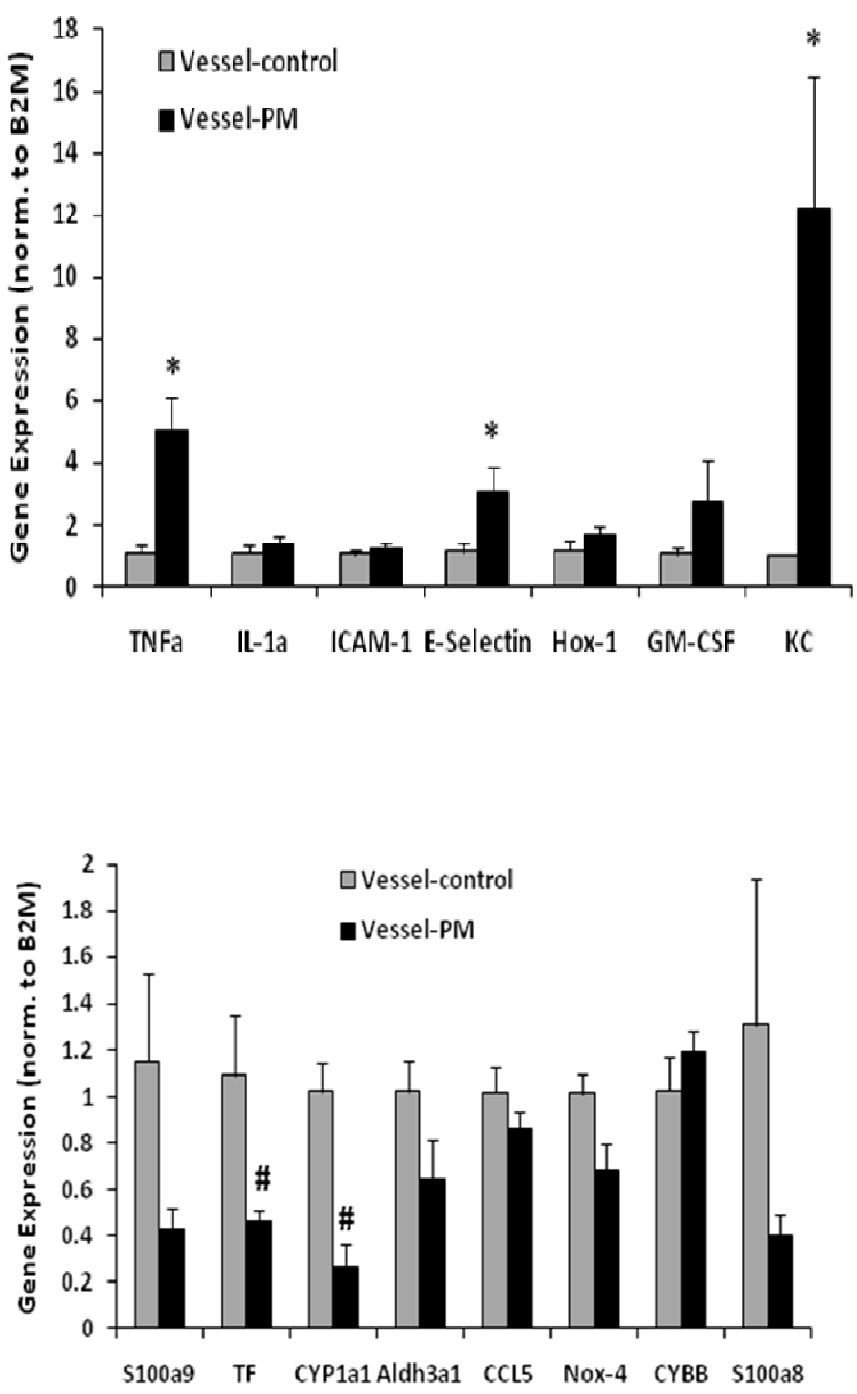


Figure 17: mRNA expression in the parenchyma of mice instilled with PM compared to controls. *p<0.05 increase, # = p<0.05 decrease



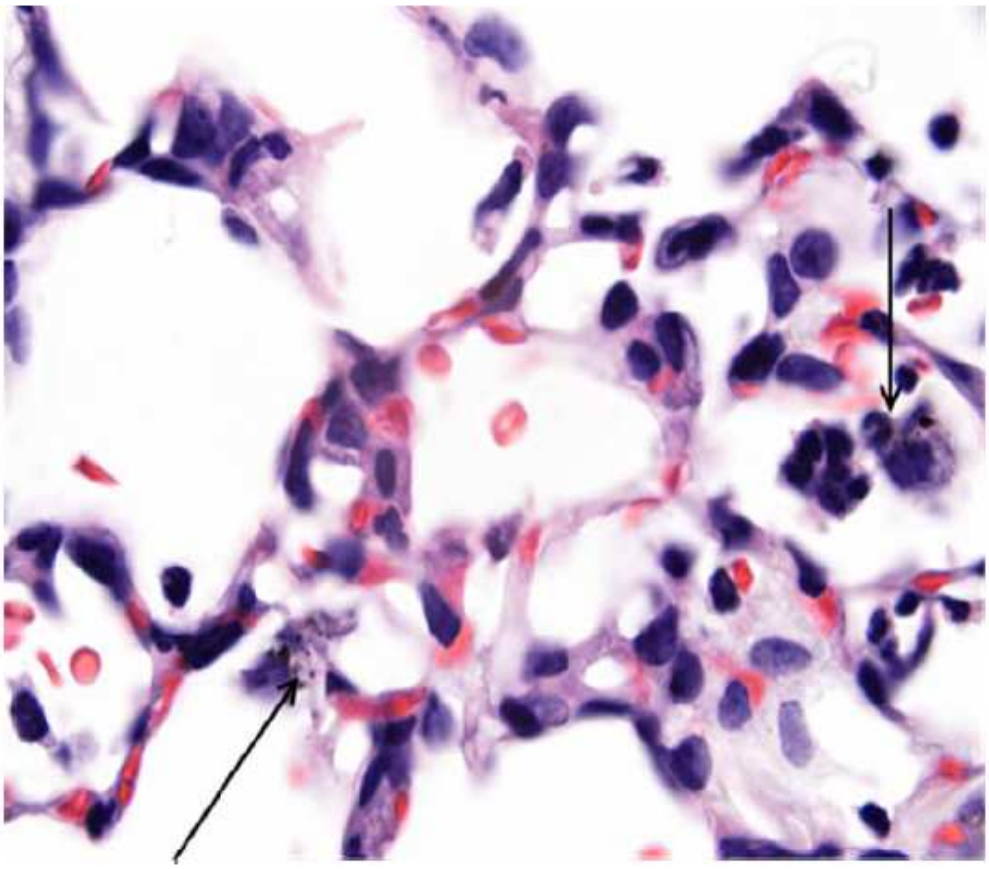
Studies of the parenchyma showed that similar to airways, there was an increase in KC (mouse homologue of IL-8), along with TNF α and that tissue factor and CYP1a1, along with CCL5, Nox-4 and S100a8 were decreased (Figure17). Evaluation of gene expression in the vessels showed a large increase in KC and E-selectin along with a decrease in tissue factor and CYP1a1. (Figure18)

Figure 18: mRNA expression in the vessels of mice instilled with PM compared to controls. *p<0.05, # = p<0.05 decrease



Histologic examination of paraffin embedded sections of lung clearly demonstrated the presence of particles in alveolar macrophages as well as the presence of increased numbers of neutrophils near or associated with the alveolar spaces (Figure 19)

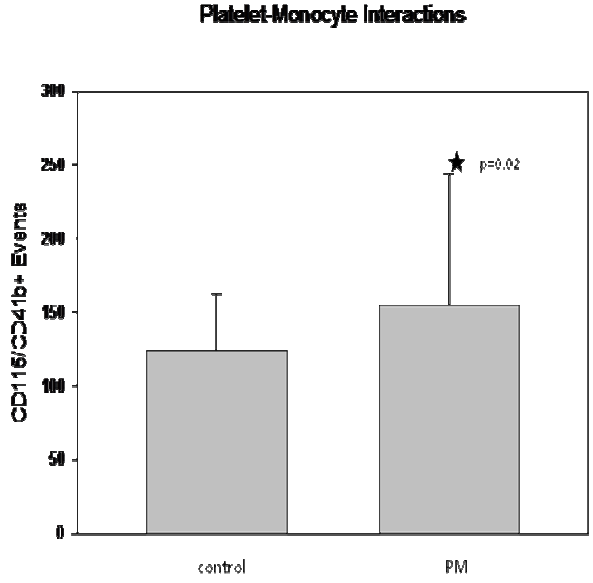
Figure 19: Section of lung. Arrows point to macrophages containing particulate matter.

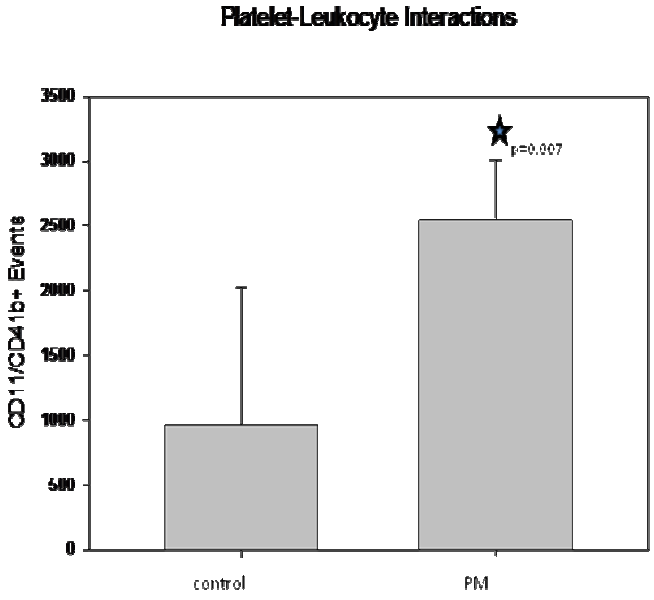


The second intra-tracheal instillation study was conducted with particles obtained from urban Fresno in Winter 2009. Flow cytometric studies showed no changes in platelet activation between the control and experimental populations. However, there were increased numbers of platelet and monocyte interactions associated with intra-tracheal instillations ($p=0.002$). (155 vs 124 cells labeled with antibodies to both CD41b and CD115). In addition, there were significantly increased numbers of platelet-leukocyte

interactions seen in the PM instilled animals (p=0.007, 2542 vs. 956 positive cells) (Figure 20)

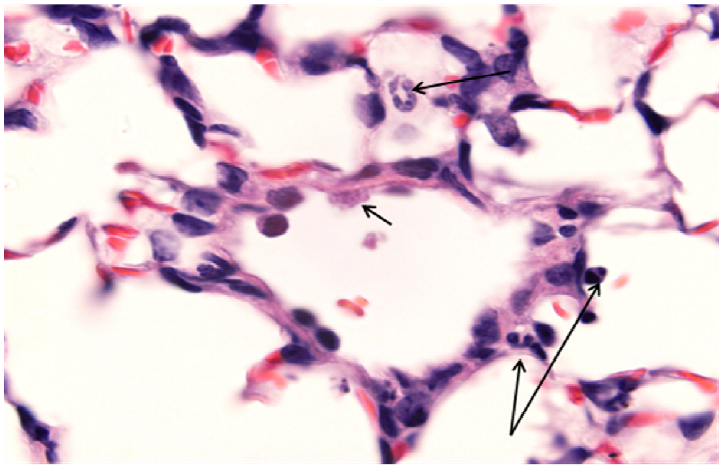
Figure 20: Interaction of platelets and monocytes (top panel) and leukocytes (bottom panel in mice instilled with urban Fresno winter 2009 PM compared to controls.





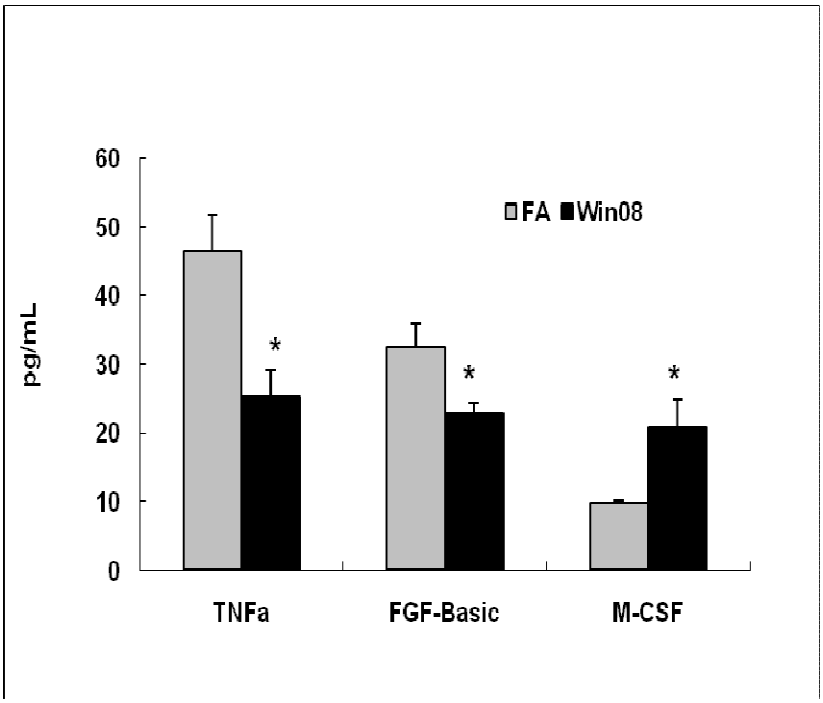
Histologic examination was conducted on lungs from both control and PM instilled animals. Observations of the PM instilled lungs demonstrated increased inflammation in areas adjacent to small arterial vessels, suggestive of a localized inflammatory response. In addition, mononuclear cells were present and closely adherent to the vascular endothelium, as were clumps of platelets. (Figure 21)

Figure 21: Section of lung. Short arrow points to a platelet clump attached to endothelial cells. Long arrows demonstrate the presence of leukocytes.



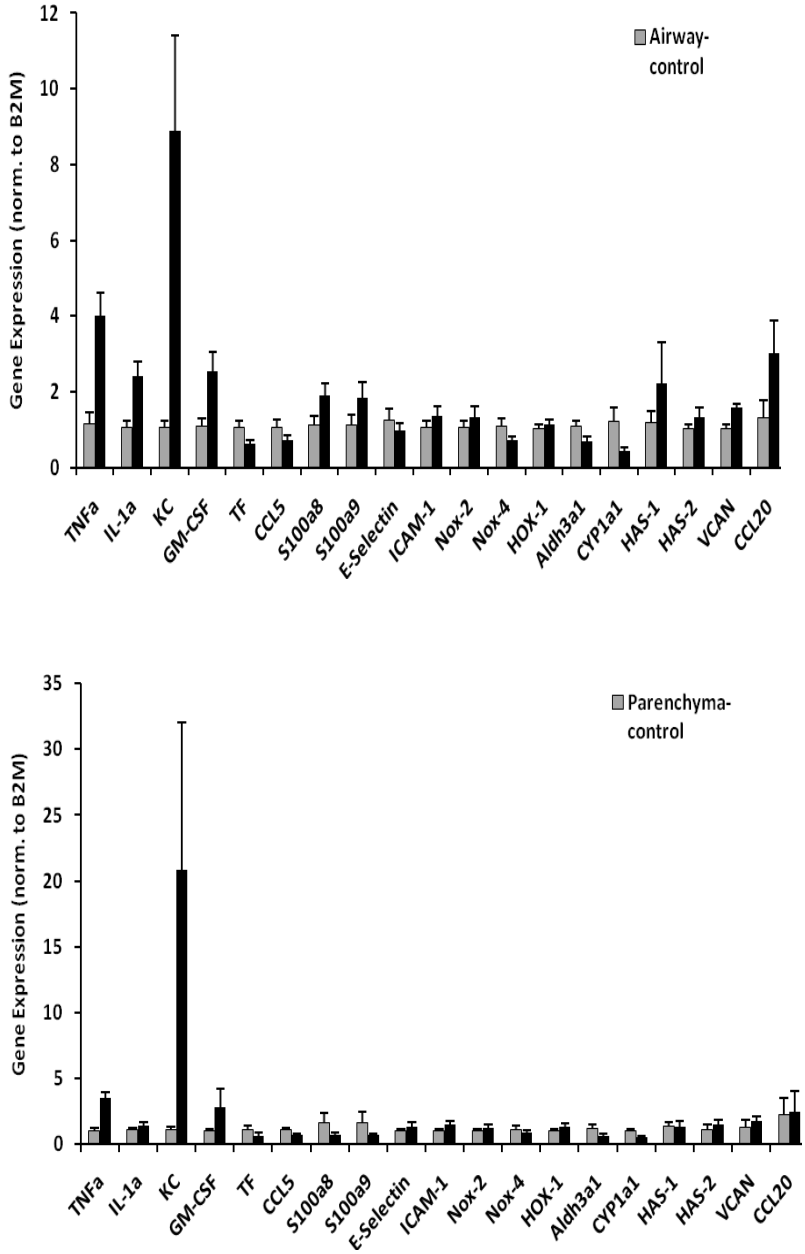
Evaluation of serum cytokines showed significant increases in MIP-1a, IL-2, Il-4 and TNFα (mean + SEM, Figure 22).

Figure 22: Serum cytokines in mice intratracheal exposed to winter urban Fresno 2009 PM compared to controls. *p<0.05

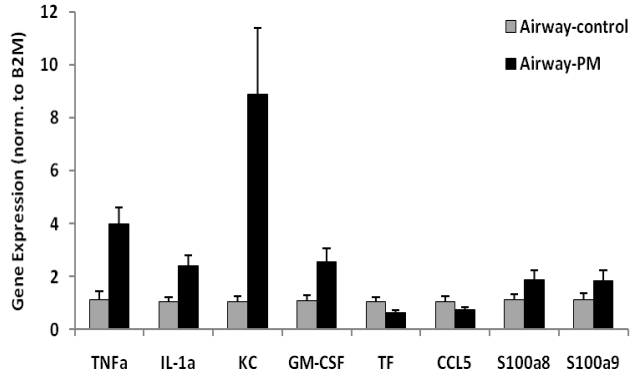


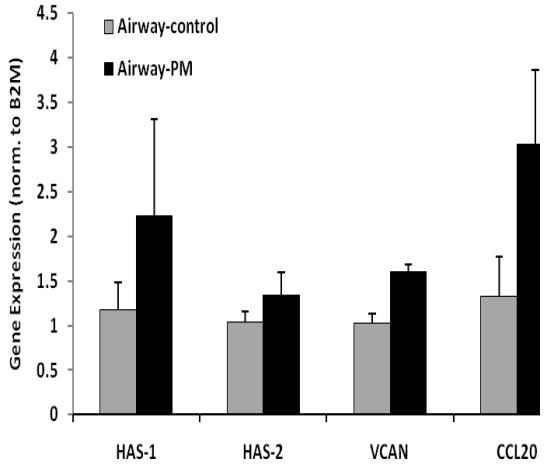
Whole lung cytokine arrays showed no changes in cytokines in the PM versus the saline instilled control animals. Laser capture analysis of airways, vessels and parenchyma demonstrated increased message for KC in all tissues (airway p=0.0011, parenchyma p=0.0047, vessels p=0.0031), as well as increased message for TNFα (airway p=0.0021, parenchyma, p=0.0007, vessel p=0.0021) Additionally GM-CSF was increased in the airways of PM treated animals (p=0.022), as well as in vessels (p-0.043) (Figure 23, mean + SEM).

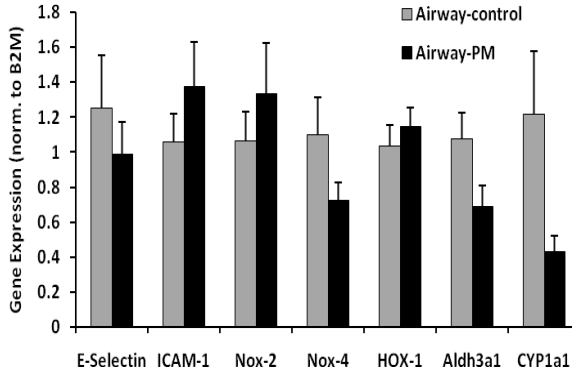
Figure 23: Laser Capture Microdissection of Airways, Parenchyma and Vessels



C:


Airway-control
Airway-PM
Gene Expression (norm. to B2M)
12
10
8
6
4
2
0
TNFa
IL-1a
KC
GM-CSF
TF
CCL5
S100a8
S100a9


Airway-control
Airway-PM
Gene Expression (norm. to B2M)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
HAS-1
HAS-2
VCAN
CCL20


Airway-control
Airway-PM
Gene Expression (norm. to B2M)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
E-Selectin
ICAM-1
Nox-2
Nox-4
HOX-1
Aldh3a1
CYP1a1

Discussion:

Cardiac events are the main source of morbidity and mortality associated with high levels of ambient PM exposure (Peters et al. 2001; Samet et al. 2000). In addition, many studies have shown that exposure to PM results in pulmonary associated oxidative stress, which is accompanied by the release of inflammatory cytokines, epithelial dysfunction and exacerbated respiratory disease (Boscia et al. 2000; Bouthillier et al. 1998; Chuang et al. 2007; Frampton et al. 2004; van Eeden et al. 2001). Our results demonstrate that a two week exposure of mice to CAPs from a geographic region with significant PM associated air quality problems results in moderate increases in pro-inflammatory cytokines, up-regulation of pulmonary gene expression, and up-regulation of platelets in the circulation. .

The observation that PM-treated pulmonary microvascular endothelial cells (MVEC) secrete pro-inflammatory factors that activate monocytes provides an alternative mechanism by which inhaled PM could induce systemic inflammation. As the next defensive barrier beyond the alveolar epithelium, the pulmonary microcirculation is the first point at which inhaled PM could be introduced into the circulation. Based on our results, we propose that this site could also allow paracrine monocyte and platelet activation by PM. A recent study by Ishii et al. 2005) showed that supernatant from PM-treated alveolar macrophages could activate bronchiolar epithelial cells, demonstrating a role for paracrine signaling in lung inflammation. In our study, the THP-1 inflammatory responses to PM were substantially higher than the responses to supernatants from PM-treated MVECs. However, the monocytic cell line THP-1 xenobiotic response to MVEC supernatants was essentially identical as direct PM treatment. Following PM treatment, the supernatants from MVECs were centrifuged in an effort to remove insoluble particulate matter. However, it is possible that ultrafine elements of the PM such as PAH remained in solution, perhaps bound to albumin present in the medium, and were inadvertently included during monocyte treatment. However, it is unlikely that endotoxin from PM is transferred to THP-1 cultures, as including PB in the supernatants prior to exposure does not reduce the inflammatory response. In summary, we have proposed a mechanism of systemic monocyte activation by inhaled ambient PM from air pollution. As PM contains a diverse array of potentially damaging components, their effects on monocytes are in turn varied. We have shown that the primary stimulus for PM-induced monocyte activation is ROS, generated modestly from chelatable transition metals directly from particles, but primarily intracellularly by endotoxin- and PAH-induced signaling mechanisms (den Hartigh et al. 2010). An additional response to PM-induced ROS production was increased CYP1a1 expression, with AhR signaling being potentially responsible for cytokine synthesis. Finally, our results suggest a novel alternative mechanism of monocyte activation mediated by paracrine signaling through pulmonary microvascular endothelial cells. Future work with human monocytes, a cell

that can be easily and routinely sampled from humans, could help to determine a population's exposure risk to environmental PM.

Most, if not all, inflammatory processes are multicellular events requiring interactions of leukocytes, endothelial cells and platelets (Bazzoni et al. 1991). Platelets are generally accepted as key mediator and effector cells of pulmonary inflammation and the allergic response (Capron et al. 1987; Page 1989; Coyle et al. 1990; Herd and Page 1994). Morley et al. (1984) demonstrated that depletion of platelets from an animal model of asthma resulted in diminished antigen dependent pulmonary infiltration of eosinophils and neutrophils. Similarly, Coyle et al. (1990, 1993) showed that platelet alpha granule products such as platelet activating factor (PAF) and platelet factor 4 (PF4) were present in the bronchoalveolar lavage of animals exposed to antigen challenges. These observations support a role for platelets as both effector and mediator cells in a variety of lung diseases (Herd and Page 1994). Our studies demonstrate that CAPs exposure results in an underlying shift in the procoagulant status of these animals reflected both in increased platelet numbers (Winter 2008) and the enhanced sensitivity of the platelets to physiological agonists (in all CAPs studies). Additionally, in our intra-tracheal studies we have clear evidence of platelet-monocyte interactions, an additional marker of a procoagulant environment.

Our studies showed strong indications of platelet activation as assessed by flow cytometry. One measure of the state of activation of the major platelet integrin, α2b-β3a is the cell's ability to bind its primary ligand, fibrinogen. In unstimulated, resting platelets the integrin α2b-β3a is in an inactive conformation, binding virtually no fibrinogen (Faraday et al. 1994; Heilmann et al. 1994). Platelet activation results in up-regulation of the integrin and enhanced fibrinogen binding. Interestingly, our Winter 2008 CAPs study demonstrated that unstimulated platelets from CAPs exposed animals bound more fibrinogen than those exposed to ambient air. In contrast, our Winter 2009 CAPs study showed that there was no change in fibrinogen binding in unstimulated platelets, however there was a 75% increase in fibrinogen binding of ADP stimulated platelets from CAPs exposed animals.

Platelet alpha granules contain a variety of chemokines which are released upon platelet activation. Clemetson and colleagues (Clemetson et al. 2000) have shown that platelets have receptors for MCP-1, RANTES, MIP-1α and MIP-1β, all of which were up-regulated in the serum of our PM exposed animals. Binding of these ligands to their platelet receptors has been suggested to act through an autocrine-feedback mechanism, resulting in the priming of platelets for activation without actually causing platelet aggregation (Gresele et al. 2008). Platelet priming has been suggested to act by sensitization of platelets such that these cells will enhance their response to stimuli (Gresele et al. 2008). Such sensitization and subsequent stimulation by physiological agonists results in the release of platelet alpha, dense and lysosomal granule contents.

Alpha, dense and lysosomal granules are all released during platelet activation. Expression of LAMP-1 on the platelet surface indicates release of lysosomal granules and is an indicator of platelet activation (Silverstein and Febbraio 1992). Our studies demonstrated that significantly greater numbers of CAPs exposed platelets from all of our field studies, expressed LAMP-1, and/or P-selectin on their surface after agonist stimulation. Increased LAMP-1 and P-selectin expression in response to agonist, increased fibrinogen binding and increases in platelet related chemokines, as in the case of CAPs exposed animals, provides evidence of platelet priming.

Activated platelets release factors that can act as signals for target cells, including the cells of the innate and adaptive immune system where they promote cell-cell interactions (Herd and Page 1994; Klinger, 1997). Among these factors, RANTES has several immune and inflammatory activities, including serving as an eosinophil chemotactic factor (Kameyoshi et al. 1992), as well as priming T cells for a TH2 response (Sallusto et al. 1998). Our intra-tracheal experiments demonstrated statistically significant platelet-monocyte interactions in PM exposed animals. When platelets adhere to monocytes and release RANTES, the platelet-monocyte complex associates with the surfaces of up-regulated endothelial cells, serving as localized signals for monocyte-endothelial cell adhesion and the development of atherogenic plaques (McIntyre, et al. 2003;Schober et al. 2002; von Hundelshausen et al. 2001). PM inhalation is associated with increased accumulation of lipid plaque in apoE knockout mice (Chen and Nadziejko 2005) and reductions in flow mediated vasodilation, (Dales et al. 2007; Peretz et al. 2008) which could be mechanistically associated with our experimental evidence of RANTES release. Platelets have been suggested to be early effectors of dendritic cell activation in tissue injury (Gallucci and Matzinger 2001) and platelet release of RANTES has been shown to have a role in the local regulation of dendritic cells (Kameyoshi et al. 1992; Weyrich et al. 1996). Platelets have also been shown to contribute to adaptive immunity (Elzey et al. 2003). In our study, increased TH-2 specific responses are suggested by elevations in RANTES, IL-13 and IL-5. In addition to TH-2 responses, IL-13 and IL-5 both stimulate eosinophil production, important modulators of the allergic/asthma response. Further contributions to a pro-asthma like response could result from the increase in PDGF-bb, which stimulates angiogenesis as well as myofibroblast and smooth muscle proliferation (Barrientos et al. 2008). IL-10 is a down-regulator of both TH-2 and eosinophil responses, and its co-induction would imply a balanced immuno-stimulatory response. Similarly, MIP-1β acts through CCR5 receptors on dendritic cells to stimulate TH-1 responses and down-regulate eosinophil activation. IL-10 also inhibits the pro-TH-1 cytokine IL-12(p40), which was the only activity that was lower in CAPs exposed animals than in filtered air controls. Interestingly, platelets also have been demonstrated to interact with dendritic cells causing the release of IL-10 as well as decreased production of IL-12 (Kissel et al.

2006), suggesting that platelets may participate in a dendritic feedback loop as part of the CAPs response.

IL-6 has been shown to play a pivotal role in hemostasis, increasing expression of tissue factor, fibrinogen (Amrani 1990), vWF, and factor VIII (Stirling et al. 1998). IL-6 also increases platelet production, activates endothelial cells and stabilizes fibrin clots (Kerr et al. 2001, Burstein et al. 1996, Neumann et al. 1997). Furthermore, it promotes coagulation through increased generation of thrombin without affecting downstream fibrinolysis (Stouthard et al. 1996). Thus increases in IL-6 may increase the risk of thrombosis and coronary artery disease (Kannel et al. 1987). PM studies by Mutlu et al. (2007) demonstrated that pulmonary macrophages are largely responsible for the production of IL-6.

Mutlu et al. (2007) also demonstrated that the airway epithelium and/or neutrophils were responsible for PM induced production of TNFα. *In vitro*, cardiac myocytes and epithelial airway cells incubated with PM secrete TNFα (Totlandsdal et al. 2008). Endothelial responses to TNFα include increased leukocyte adhesion, increased procoagulant activity and release of secondary cytokines including PDGF.

The effects of the variety of chemokines demonstrated in this study suggest a potential role for endothelial activation in response to PM inhalation and/or pulmonary inflammation resulting in up-regulation of endothelial cell surface receptors known to activate platelets. Our laser capture studies demonstrated several different pathways of endothelial up-regulation dependent upon the various CAPs or intra-tracheal exposures. Several experimental conditions have demonstrated an increase in expression of E-selectin and TNFα. Additionally we have demonstrated increases in NOX-2 and ICAM-1. In all of these experimental conditions, we also noted increases in inflammatory cytokines, notably TNFα and MIP-1α and MCP-1.

Furthermore, the moderately elevated concentrations of PAHs present in our CAPs exposures have previously been shown to result in increases in pulmonary bronchial epithelial cytokines (Happo, et al. 2008, Goulaouic et al. 2008, Pei et al. 2002). However, other particle characteristics are likely involved in the inflammatory response. Traditionally, cytochrome P450 enzymes were thought to be expressed primarily in hepatic cells, where their major function is to metabolize drugs, toxins, and endogenous waste products. More recently, it was found that CYP1a1 expression is much higher in whole lung tissue than in liver (Yamamoto et al., 2004), and as such CYP1a1 has become an important target to examine pulmonary effects of diesel exhaust and air pollution particles. Increases in xenobiotic metabolism noted in our laser capture and lung gene array studies, evidenced by increases in CIP1-a1 and Aldh3a-1, as well as studies by denHartigh et al (2010) from our laboratory has shown that high level of CYP1a1 expression are observed after PM exposure, likely due to the presence of

PAH. These compounds are commonly found in diesel exhaust and air pollution particles and possess mutagenic and carcinogenic properties, mediated by their affinity to intracellular receptors such as AhR. Thusly, we suggest that PAHs present in our CAPs exposures may contribute to local alveolar inflammation.

Interestingly, a recent study found many of the activities increased in our study were regulated by the interactions between epithelium and endothelium in combined *in vitro* studies and models of response to airway injury (Zani et al. 2008). Direct interaction of PM with the pulmonary microvasculature could also initiate platelet-endothelial activation. One study investigating this showed upregulation of the pro-coagulant activity of Tissue Factor (TF) and correlative down regulation of thrombolytic activity of tissue plasminogen activator (tPA) in PM treated umbilical vein endothelium (Gilmour et al., 2005). A recent study also demonstrated release of tissue factor, from cultured bronchial airway epithelial cells exposed to collected ambient PM2.5 (Sun et al. 2008) This suggests an additional alternative that PM induced airway epithelial cell cytokine release could directly prime platelets. It seems likely that integrated responses of multiple pulmonary cells will best explain the cascade of events leading to systemic platelet activation.

The specific mechanisms by which inhaled ambient particulate matter induces systemic inflammatory responses remain an enigma, although the results of this study provide evidence that helps to explain how inhaled PM2.5 affects the cardiovascular system. While it is relatively well established that small amounts of inhaled ultrafine particulates enter the blood (Nemmar et al., 2002), the circulation time is transient and the concentrations achieved are low enough to generate much uncertainty as to their significance. An alternative explanation could be that the alveolar microvasculature provides a site of higher particle concentration that could activate circulating inflammatory cells either by direct contact in the low flow environment or in a paracrine fashion through activation of microvascular endothelium. Fine particulate matter is readily taken up and cleared from the lungs by alveolar macrophages, leaving behind accumulated ultrafine particulates (Takenaka et al., 2001).

Several groups have reported that CYP1a1 and NFkB behave antagonistically towards each other, with the belief that stimuli for the NFkB pathway promoting inflammatory responses decrease the CYP1a1 response, and vice versa (Ke et al., 2001). As such, it was recently suggested that there are two distinct pathways that involve CYP1a1: the genomic pathway and a non-genomic pathway in which ARNT is not involved (Sciullo et al., 2009). The non-genomic pathway is characterized by a rapid inflammatory response, followed by the characteristic CYP1a1 expression after 3 h of stimulus. It has been proposed that certain PAH compounds, such as nitro-PAH, can induce heterodimerization of AhR with RelB, a component of the NFkB family of transcription factors (Ovrevik et al., 2009). This would in turn initiate an inflammatory pathway

hinging on NFkB activation, rather than the classical genomic pathway of CYP1a1 expression. In the present study, the relative abundance of both inflammatory cytokines and CYP1a1 mRNA after 3 h of PM treatment suggests that AhR and NFkB were both activated by our PM exposures. In addition, the observation that polymixin B attenuated PM-induced cytokine expression that is known to be NFkB-dependent, but significantly amplified AhR dependent CYP1a1 expression suggests that by disrupting NFkB signaling, more activated AhR was directed towards xenobiotic response elements, thereby increasing CYP1a1 expression. A potential starting point for both the genomic and non-genomic pathways leading to cytokine and CYP1a1 expression is ROS. PAH compounds such as B[a]P that induce the classic genomic pathway of AhR activation are also known to induce oxidative stress in macrophages (Fabiani et al., 1999,. Li et al. 2008) suggest that ultrafine PM, which is often rich in PAH, stimulates the production of ROS, which in turn initiates inflammatory signaling pathways involving NFkB. Our finding (den Hartigh et al., 2010) that blocking AhR activation with α-naphthoflavone did not reduce intracellular ROS suggests that PAH present in our PM stimulates ROS production before the AhR signaling pathway is initiated.

The results presented in the den Hartigh et al. (2010) study suggest that ROS are intimately involved in both xenobiotic and inflammatory gene expression. Specifically, we have shown that neutralizing transition metals using DFM and inhibiting intracellular ROS using NAC resulted in decreased cytokine and CYP1a1 gene expression when compared to PM treatment, while Catalase had no effect on either. While we did not measure particle-generated ROS directly from our PM, others have shown that ambient particles collected from southern California contain measurable ROS originating primarily from copper ions (DiStefano et al., 2009). The fact that NAC completely attenuated the intracellular cytokine and CYP1a1 expression in response to PM, while Cat had no effect and DFM had a minimal effect, suggests that ROS generated intracellularly in response to PM are primarily responsible for these endpoints.

Summary and Conclusions

Our data suggest that there are significantly different responses to particles from summer exposures than from winter exposures. Winter exposures, whether to CAPs or intra-tracheal exposures, resulted in a greater pro-inflammatory state, which was reflected in increased platelet sensitivity to agonist, increased pro-inflammatory serum cytokines, and increases in CYP1A1 and TNF-alpha expression in the lung. Increased expression of CYP1A1 throughout the lung, maybe due to increased levels of PAHs shown to be present in winter exposures. Increased expression of TNF-alpha generally reflects a pro-inflammatory microenvironment, however, it is not clear which component of PM may be responsible for this up-regulation.

Recommendations

Given the information generated from our studies, it will be critical to dissect the contributions of trace metals, PAHs and endotoxin in PM to better understand how these components affect the cardiovascular response to air pollution. It appears, based on the studies presented herein, that exposure to PM with high levels of PAHs and/or metals or endotoxin results in an increased pro-coagulant environment and inflammation as defined by platelet activation and release of pro-inflammatory serum cytokines.

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